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THE AGGLUTINATION TEST IN VIBRIONIC ABORTION OF SHEEP

By

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INTRODUCTION

BLAKEMORE and Gledhill (1946) reviewed the literature on vibronic abortion and reported investigations on four outbreaks in East Anglia. Using merthiolated suspensions of one motile strain of *Vibrio foetus* they found that sera from 20 normal sheep gave titres of 1 in 20 or less, whilst most of those from infected animals agglutinated the suspensions to higher dilutions. The differences in titres of infected and normal sheep sera were, however, neither wide enough nor constant enough to permit a reliable interpretation of the test; this was especially so when the bacterial suspension was not made from the strain of vibrio associated with the outbreak. Evidence was also presented from experiments with rabbits that, whilst merthiolated suspensions of two strains were antigenically related, alcohol-treated suspensions of three strains were not so. Blakemore and Gledhill's work required confirmation because of the small number of strains they investigated, and because exception might be taken to their use of broth containing 5 per cent. horse serum in the preparation of suspensions intended for the agglutination tests and for the immunisation of rabbits. This paper records the examination of further strains of *V. foetus* and includes some incidental observations regarding the maintenance and preservation of the organism in artificial culture.

MATERIAL AND METHODS

Five strains of *V. foetus* labelled B, M, G, W and F were isolated in 1945 from different outbreaks of abortion in ewes in Berkshire (B, M, G), Oxfordshire (W), and Sussex (F). They were isolated and subcultured on a serum-free, liver agar medium and incubated at 37° C. in an atmosphere enriched with CO₂. All the strains were micro-aerophilic on isolation. Growth and motility were good with strains B, G, W and F and less with strain M. Suspensions for agglutination tests were prepared from cultures on liver agar made as follows:—450 g. of minced beef liver was suspended in a litre of tap water and steamed for 3 hours. It was allowed to cool and passed through grey filter paper and the filtrate enriched with 1 per cent. Difco or Evans' peptone, 0.5 per cent. sodium chloride and 2 per cent. shredded agar. The pH was adjusted to 7.8 and the medium was then autoclaved at 120° C. for 15 minutes, filtered through wet paper pulp, readjusted to pH 7.2 to 7.4, distributed in Roux bottles and autoclaved again at 110° C. for 15 minutes.

The growth obtained after inoculating the freshly prepared

bottles of solid medium with 24 hour broth subcultures and incubating for 48 hours in 10 per cent. CO₂ was tested for motility and purity, after which it was washed off with saline. The suspensions made up to an opacity corresponding to that of tube No. 1 of MacFarland's scale were preserved with Formalin Analar (final concentration 0·2 per cent.) and stored in the dark at room temperature. Only stable suspensions were used for the agglutination tests against sheep or rabbit sera. In these tests 1 ml. quantities of serial two-fold dilutions of serum from 1 in 10 upwards were mixed and shaken with equal amounts of suspension and the mixtures incubated at about 54° C. in a water-bath overnight. Readings were recorded as complete, almost complete, slight and negative and the titre of a serum was arbitrarily chosen as the highest dilution in which almost complete agglutination occurred.

The titres of 52 normal sera from three abortion-free flocks in Berkshire (Table I) were compared with 57 sera from ewes that aborted in the five outbreaks (Table II). Aborted ewes in flocks B, G, W and F were bled two to four weeks after the onset of abortions, whilst in those of flock M the lag period was about eight to ten weeks. The serum of each aborted ewe was tested against a suspension of its homologous strain and some of the samples were tested also against heterologous strains.

Immediately after isolation the five strains were used to immunise rabbits—one rabbit per strain—for the preparation of agglutinating sera. Motile cultures in broth, which had been incubated for 24 to 48 hours and were sometimes supplemented with cultures on liver agar, were inoculated intravenously on five to seven occasions at intervals of a few days. The animals were bled 10 to 14 days after the last injection and the sera, preserved with an equal volume of glycerol, were stored at 4° C. For comparison a sixth rabbit was similarly immunised with a recently isolated strain of *V. foetus* from a bovine foetus. The sera were tested against homologous and heterologous suspensions (Table III) in the same way as sera from normal and infected sheep.

RESULTS

Table I shows that agglutinins are present in sera from normal sheep. The influence of the strain used in the preparation of the agglutinating suspension is obvious. Thus with strains G, F and W the critical titre could be assumed to be in the region of 1 in 40 or less, whilst with similar suspensions made from strains B and M it was 1 in 80 and 1 in 160 respectively. These titres were obtained whether the tubes had been incubated at 54° C. or at 37° C. and preliminary heating of the sera to 56° C. for 30 minutes did not significantly alter them. Older animals usually had higher titres than young ones—a well-known observation with many bacterial suspensions.

In general, sera from ewes that had aborted agglutinated the

TABLE I
AGGLUTININS IN 52 NORMAL SHEEP SERA TESTED AGAINST FORMALINISED
SUSPENSIONS OF *V. foetus*

Suspension made from strain	Number of sera tested	Number of sera reacting at various dilutions				
		1:10*	1:20	1:40	1:80	1:160
B	12	1	—	2	9	—
M	10	—	—	3	5	2
G	52	46	4	2	—	—
W	10	8	1	1	—	—
F	37	24	4	9	—	—

* 1:10 or less.

homologous (infecting) strain above the maximal titre obtained with sera from normal sheep (Table II). This was the case with 46 out of 57 sera tested. Five of the eleven false negative reactions were observed in sera from ewes M that had been bled 8 to 10 weeks after abortion; Blakemore and Gledhill (1946) reported that a delay was accompanied with a definite loss of titre. When suspensions of heterologous strains were used, *i.e.*, those isolated from other outbreaks, the incidence of false negative results increased. Of 49 tests with heterologous suspensions, no less than 24 were negative or doubtful. Blakemore and Gledhill (1946) recorded similar findings with seven sera from aborted ewes; they obtained titres of 1 in 160 or above with the homologous strain (VT), but with another strain (VS), which they had recovered from a separate outbreak, the reactions were more irregular and generally occurred to a lower titre. Whether the difference observed in their case was really significant is by no means clear, as these authors appear to have determined the agglutinin titre of normal sheep serum against one of these strains only (VS).

The experiments on rabbits (Table III) showed that the discrepancy between the results of tests carried out with homologous and heterologous suspensions could be accounted for partly by the difference in antigenic structure of the strains used. The sera of

TABLE II
AGGLUTININS AGAINST HOMOLOGOUS STRAINS IN SERA FROM ABORTED EWES

Out- break	No. of sera tested	Number of sera reacting at various dilutions								Critical titres†
		1:10*	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
B	12	—	—	—	1	—	3	4	4†	Over „ 1:80
M	13	—	—	1	4	8†	—	—	—	„ 1:160
G	9	—	—	1	1	3	2	1	1†	„ 1:40
W	13	—	—	—	7	2	2	2	—	„ 1:40
F	10	2	1	1	—	3	2	—	1†	„ 1:80

* or less. † Sera not tested beyond that dilution. ‡ See Table I.

six rabbits immunised with live motile cultures from six strains (five ovine and one bovine) agglutinated to high titres formolised suspensions of the homologous strains, but not always those prepared from heterologous strains. Significant cross-reactions were observed, such as those between B and G, or G and W, or W and F. The results suggested a partial antigenic relationship among those groups of strains rather than complete antigenic identity. Agglutinin-absorption tests which were carried out with serum F alone confirmed this. The agglutinins in serum F were completely

TABLE III
AGGLUTININS IN SERA FROM RABBITS PREPARED AGAINST VARIOUS STRAINS
OF *V. foetus*

Antiserum	Titres obtained with suspensions of various strains				
	B	M	G	W	F
B	1 : 2,560	—	1 : 1,280	1 : 20	—
M	—	1 : 1,280	1 : 80	—	—
G	1 : 640	—	1 : 5,120	1 : 20	—
W	—	—	1 : 640	1 : 10,240	1 : 640
F	—	—	—	1 : 640	1 : 2,560
H*	—	Not tested	160	—	—

* Serum prepared against a strain of *V. foetus* isolated from an aborted cow. Its titre against the homologous organism was more than 1 : 2,560.

— = Negative.

absorbed with a thick living suspension of strain F, but not with those of B, M or G; the result obtained with a suspension of strain W was not satisfactory.

The differences in antigenic structure suggested by Table III in which immune rabbits' sera were used were, in the main, similar when the tests were made with sera from aborted ewes but this was not always the case. For example, some of the ewes from outbreaks of abortion B and F reacted significantly with suspensions from F and G respectively. The specificity of those unexpected reactions was not investigated; it is possible that sheep infected with different vibro strains may unmask certain common antigens which artificially immunised rabbits are unable to do.

GROWTH AND MAINTENANCE OF *V. Foetus*

The strains used were found to vary in their survival *in vitro* on artificial media and a combination of methods has been necessary to maintain them alive for the last four years. When first isolated, the strains were more labile than after they had been "hardened" by several sub-cultures *in vitro*. Their periods of survival under different conditions are recorded in Table IV. In cooked meat medium and in stomach contents of aborted fetuses, covered, in each case, with a seal of liquid paraffin, the temperature of storage appeared to be important. Comparative tests carried out under

these conditions have confirmed the observations of earlier workers that the organism will survive for longer periods at room temperature than in the refrigerator (Smith and Taylor, 1919).

TABLE IV
COMPARATIVE VIABILITY, IN MONTHS, OF STRAINS OF *V. fetus* PRESERVED
BY DIFFERENT METHODS

Method of preservation	Strain				
	B	M	G	W	F
Fluid stomach contents of aborted fetus covered with liquid paraffin ...	>24	Not done	>20	Not done	Not done
Cooked meat broth or liver agar covered with liquid paraffin ...	2-12	1-3	2-18	2-18	2-18
Drying— <i>in vacuo</i> ...	7-12	>36	7-12	7-12	7-15

> More than.

Freeze-drying of bacterial growth suspended in a few drops of 2 per cent. horse serum broth *in vacuo* over P_2O_5 and replacing the vacuum after 24 hours with nitrogen proved very useful. Proom and Hemmons (1949) have also obtained long survival (four years or over) by this method with two stock strains of *V. fetus* and though the results recorded here are not so good as theirs it is possible that improvements in drying technique would enable us to obtain better, and particularly more consistent, survival.

DISCUSSION

The agglutination test in vibronic abortion of sheep is beset with difficulties partly associated with the preparation of the bacterial suspension and partly with the poor agglutinin response by the host. Strains of *V. fetus* appear to differ in their growth requirements. The five sheep strains that were studied in this paper and two others of more recent isolation were found to grow fairly well on the liver agar described earlier. The growth of strain M was not as profuse as that of the other strains, but satisfactory yields have been obtained. Such was not the case with two strains isolated from bovine fetuses and it is possible that some sheep strains obtained from certain flocks may react likewise. Some workers (Blakemore and Gledhill, 1946; Brocklehurst, 1946) used serum-enriched broth or agar, but these media are not to be advised as fresh serum, especially that from species with normal agglutinins (sheep, cattle and horse), might have undesirable effects on the specificity of resulting suspensions.

The variations in the titre of the normal agglutinins observed when suspensions made from different strains were used do not appear to have been stressed by earlier workers. Within limits, such variations are known to be of common occurrence with strains of

other species of bacteria and ovine strains of *V. foetus* are no exception. In contrast, however, the latter also show major qualitative differences in antigenic structure and these are revealed in tests with sera from infected ewes and rabbits. Strains of *V. foetus* of bovine origin would appear to be serologically more uniform than those isolated from sheep judging by the report of Smith and Taylor (1919), who found that 23 out of 24 bovine strains showed definite cross-agglutination. Nevertheless, as all of these were recovered from fetuses and calves in one large herd, it is possible that their results may not be truly representative of strains from different herds although the findings of Plastringe and Williams (1943) tend to confirm their observations. In the present work formalinised suspensions from different ovine strains were heterogenous. This does not rule out the possible presence of common antigenic factors that could be revealed by special methods such as those used by Gardiner and Venkatraman (1935) and others in the antigenic analysis of *V. cholerae*. This aspect requires further study, but in the meantime the limitations of the agglutination test in the routine diagnosis of vibrionic abortion of sheep are stressed.

CONCLUSIONS

Formalinised suspensions made from five strains of *V. foetus* isolated from separate outbreaks of abortion were agglutinated by sera from normal sheep to different titres varying from 1 in 40 to 1 in 160; the titres obtained varied according to the strain used in the preparation of the bacterial suspension.

Sera from infected ewes were tested against their homologous strains and 46 of 57 examined agglutinated the suspensions to higher titres than normal sera. In a large number of cases (24 out of 49 tests) the sera of the infected ewes failed to react significantly when tested against suspensions prepared against strains other than the one with which they were infected. Agglutination tests with rabbit antisera suggested that the divergent results obtained with sheep sera are due to differences in the antigenic structure of strains of *V. foetus*.

The results obtained indicate that at present the agglutination test cannot be recommended as a routine method of diagnosis in vibrionic abortion of sheep.

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STUDIES ON EXPERIMENTAL STREPTOCOCCAL MASTITIS

1. INOCULATION OF LARGE NUMBERS OF *Streptococcus agalactiae*
INTO THE TEAT CANAL OF GOATS

By

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INTRODUCTION

THE discovery in recent years of potent anti-streptococcal drugs has directed the great volume of research on bovine streptococcal mastitis along therapeutic lines. Most of this work has been carried out under field conditions on natural cases of the disease and considerable success in the mass control of the disease has been achieved. However, much fundamental knowledge of bovine streptococcal mastitis remains undetermined. The expense of the cow as an experimental animal and the sheer bulk of its udder have hampered investigation of the behaviour of *Str. agalactiae* *in vivo* and of the pathological processes with which it is associated. These obstacles have been largely responsible for the lack of precise control in many experimental attempts to produce mastitis by inoculation of *Str. agalactiae*, and there is still doubt as to the exact part played by this organism in causing the disease; indeed, recent reviews of the literature (Little and Plastring, 1946; Lancaster and Stuart, 1949) show that in some instances the experimental introduction of *Str. agalactiae* into the teat canal has failed to produce clinical mastitis.

These studies, of which this is the first, were prompted by the present lack of knowledge of a reliable method of using *Str. agalactiae* to produce experimental mastitis; their object is to create at will a form of mastitis clinically, bacteriologically, and biochemically similar to naturally occurring bovine streptococcal mastitis, and thereafter to investigate the progress and pathology of the disease.

This first paper, which follows initial studies on the behaviour of *Str. agalactiae* in mice (Pattison, 1948 & 1949), is concerned with the production in goats of streptococcal mastitis by inoculation of large numbers of living *Str. agalactiae*. Goats were used in order to avoid the expense and practical difficulties of using cows and because the goat is known to be naturally susceptible to streptococcal mastitis (Bryan, 1942).

While not necessarily disagreeing with the assumption that only relatively small numbers of *Str. agalactiae* are encountered by the cow in natural circumstances, we decided to begin our studies on the experimental production of mastitis in goats by using the largest dose we felt it practicable to handle. Our aim in using what may be considered a very large dose of living organisms was to force

physiological and histological changes in order to prove the usefulness of our clinical, biochemical, and histological tests for the presence of mastitis; at a later date those tests found most sensitive could be employed with confidence when smaller infecting doses were used.

CLINICAL OBSERVATIONS AND METHODS

General. Cross-bred, pregnant goats were purchased and were housed in loose-boxes and fed on hay, greens, and concentrates. All were under observation for several months and milk yields were recorded from the time of parturition. The animals were infected approximately two months after kidding. During the week before infection a clinical examination was made twice daily, milk was tested bacteriologically and biochemically once daily, and three blood samples were taken for haematological examination. After infection, clinical examinations and bacteriological and biochemical examinations of the milk were carried out twice daily. Several blood samples were taken during the first 24 hours after infection, and thereafter selected haematological tests were made once daily.

Clinical examination. The general condition of the animal was considered and particular attention was paid to the udder. Rectal temperatures and heart rates were recorded.

Milk yield. The milk yield for each gland of each goat was recorded at morning and evening milkings from the time of parturition until completion of the experiment.

Bacteriological examination of milk. Milk samples were collected, using the aseptic technique described in the Imperial Bureau of Animal Health Report (1944). After thorough mixing, 0.02 ml. of milk was spread on bactotryptose ox-blood agar and incubated at 37° C. for 24 hours. Colonies morphologically resembling *Str. agalactiae* were subjected to the precipitin test, using Lancefield's group-B antiserum (Imp. Bur. Anim. Hlth., 1944).

Biochemical examination of milk. *Chlorides.* The chloride content was determined by titration and mercuric nitrate solution, of a tungstic acid-milk filtrate, in the presence of diphenylcarbazone as indicator (Schales and Schales, 1941; Rowland, 1949).

Whiteside test. The modified method of Murphy and Hansen (1941) was used, in which one drop of N/1 NaOH was mixed with five drops of milk and flocculation noted 30 seconds later.

Sediment volume. 50 ml. of milk were spun at 1,500 r.p.m. for 20 minutes and the volume of leucocytes and cellular debris was measured by the Tromsdorff method described by Hucker (1933). *Haematological methods.* General methods were those described by Holman (1946).

Erythrocytes. Dilutions for the red cell count were prepared with a Piney pipette. Oxyhaemoglobin was estimated with an

M. R. C. photometer. Packed cell volume was measured in Wintrobe's tubes.

Leucocytes. Dilutions for the white cell count were prepared with a Pincey pipette. Two hundred cells were differentiated in pre-inoculation samples, but after inoculation only 100 cells were differentiated as a routine, more cells being counted when the result was doubtful.

Other tests. For coagulation time capillary tubes were suspended in water at 40° C. To measure fragility the same dilutions were used as in the oxyhaemoglobin estimation, except that 0.7 per cent. NaCl was used as diluent instead of weak alkali. In the alcohol turbidity test of Love and Mawson (1948) 0.2 ml. of plasma was added to 4.0 ml. of 15 per cent. alcohol. The sedimentation rate was estimated by mixing 0.02 ml. of blood with 1.0 ml. of saline in a Wintrobe tube. The specific gravity of blood plasma was estimated by chloroform-benzene mixtures.

RESULTS OF TESTS ON GOATS DURING PRE-INOCULATION PERIOD

Before exposing goats to infection with *Str. agalactiae*, normal values were obtained from a group of 17 animals. These values are recorded in Table I and indicate the degree of variation in normal

TABLE I
PRE-INOCULATION MEASUREMENTS ON EXPERIMENTAL GOATS

Measurement	No. of animals	Average	Standard deviation ±	Maximum admissible difference (95 per cent.)
Whiteside test	14	0	0	0
Sediment volume (per cent.) ...	14	0.2	0.15	0.33
pH	13	6.7	0.12	0.24
Chloride content (mg. per 100 ml.)...	14	160	14.7	12.8
Leucocyte count (10 ³ per c.mm.) ...	14	7.2	3.52	4.70
Neutrophil adults (per cent.) ...	14	53.0	9.99	9.14
Monocytes (per cent.)	14	5.7	3.13	6.55
Lymphocytes (per cent.)	14	37.3	11.00	18.80
Eosinophils (per cent.)	14	3.2	2.48	5.15
Absolute No. of neutrophils (per c.mm.)	14	3,958	2,396	3,569
Erythrocytes (10 ⁶ per c.mm.) ...	14	12.1	1.84	2.42
Haemoglobin (g. per 100 ml.) ...	14	9.7	1.17	2.70
Packed cell volume (per cent.) ...	14	25.0	2.92	4.40
Comparative fragility (per cent. in 0.7 per cent. NaCl)	14	60.0	12.78	12.01
Alcohol turbidity (mg. protein per 100 ml.)	14	74.0	29.40	43.22
Sedimentation rate in saline (mm. per hour)	14	1.5	0.39	1.60
Specific gravity of blood plasma ...	14	1.03	0.007	0.000
Temperature (a.m.)	17	101.8	0.40	0.90
" (p.m.)	17	102.5	0.34	1.04
Heart rate (a.m.)	16	120	19.7	45.0
" (p.m.)	13	110	13.1	65.7

readings. It will be appreciated that figures of this type are merely a guide to what the normal state during a particular period of lactation may be and that individual variations within these limits of group normality may be pathological in individual animals. To be in a position to measure such variations, we have calculated the Maximum Admissible Difference, 95 per cent. (Holman, 1944) for daily variation.

There was a general tendency for temperatures to be higher in the afternoon than in the morning. Heart rates varied so much in different circumstances that they did not form a useful clinical guide.

Milk yields were seldom the same from both halves of the same udder, and one gland usually gave a consistently greater yield than the other. Care was taken to provide a standard quantity and quality of food to reduce natural variation in milk yield.

Haemolytic staphylococci were recovered regularly from one gland only of each of three of 16 clinically healthy goats examined; milk from these glands was slightly and rather irregularly abnormal as judged by the Whiteside, sediment volume, and chloride percentage tests. In all other milk samples examined no bacteriological or biochemical abnormality was recorded.

THE INOCULATION OF A LARGE DOSE OF *Str. agalactiae* INTO THE TEAT CANAL

Strain of Str. agalactiae used for infection

The strain of *Str. agalactiae*—S 13—used for infection was originally isolated in 1943 from a case of bovine mastitis; its serological and biochemical reactions and its pathogenicity for mice have been described elsewhere (Pattison, 1948, 1949). During these experiments with goats the strain was maintained by weekly subculture on bacto-tryptose ox-blood agar.

Preparation of doses for inoculation

Strain S 13 was grown in 300 ml. of Todd and Hewitt's broth (Todd and Hewitt, 1932) for 18 hours at 37° C. This culture was then centrifuged and the organisms were resuspended in 10 ml. of the broth. Viable counts, made by surface plate counts on bacto-tryptose ox-blood agar, showed the dose to be in the order of 100,000 million living organisms. The same volume of control inoculum of killed organisms was prepared by heating in a water bath at 75° C. for one hour, and of culture filtrate by filtration through a Seitz EK sterilising pad. Examination of control inocula showed that none contained living organisms.

Administration of dose

Infecting and control doses were introduced into the teat canal beyond the sphincter through a smooth-ended No. 18 S.W.G. needle attached to a hypodermic syringe. After the animal had been seated

on its haunches the legs were held firmly, and the udder and teats were washed thoroughly with soap and water and swabbed with absolute alcohol before inoculation.

Duration of experiment

In this first experiment our object was to detect the early and, possibly, most acute changes after inoculation; for this reason the animals were kept under observation for only nine days.

Plan of experiments

The experiments were conducted in three stages (Table II).

TABLE II
PARTICULARS OF GOATS AND INOCULA

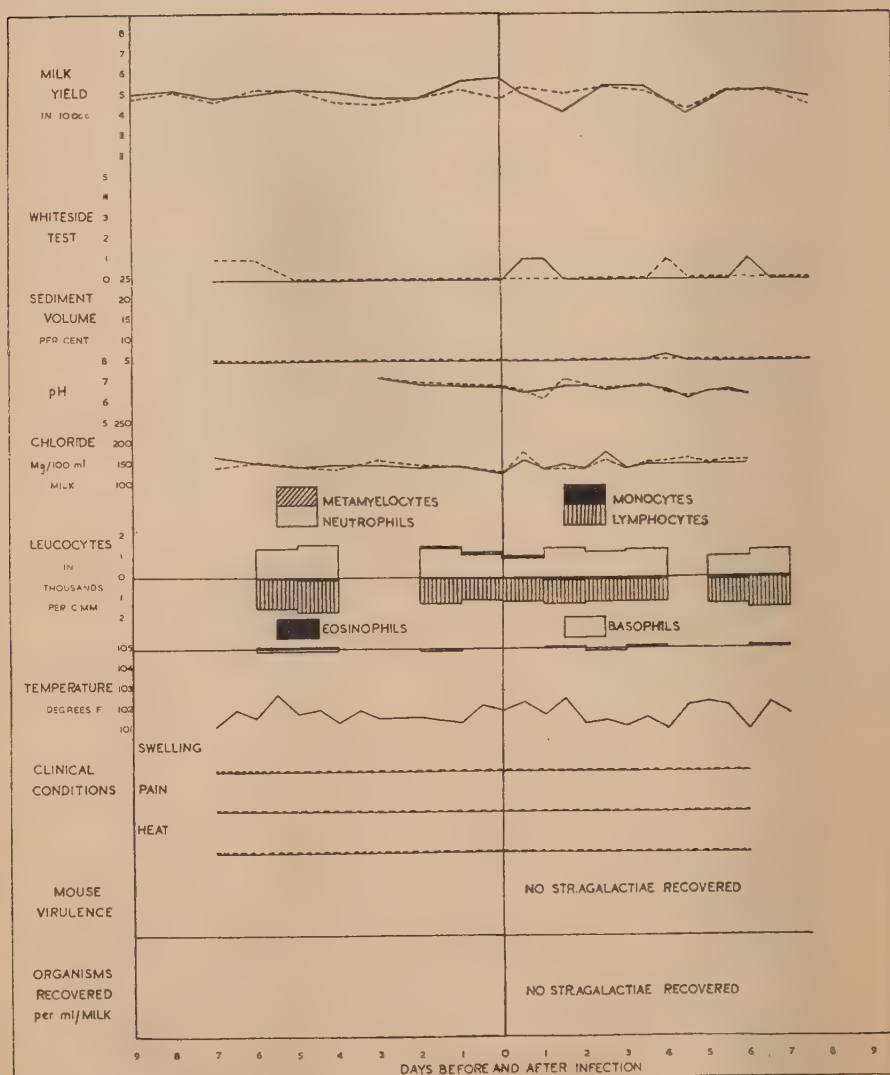
Experi- ment No.	Goat No.	First half of udder			Second half of udder		
		Inoculum	Volume of inoculum	No. of living organisms in inoculum	Inoculum	Volume of inoculum	No. of living organisms in inoculum
1	1	Whole broth culture	10.0 ml.	130,000 millions	Seitz EK filtrate of whole broth culture	10.0 ml.	Nil
2	2	Whole broth culture	10.0 ml.	88,000 millions	Nil	Nil	Nil
	3	"	"	"	"	"	"
	4	"	"	"	"	"	"
	7	Heat - killed whole broth culture	"	Nil	"	"	"
	9	Seitz EK filtrate of whole broth culture	"	Nil	"	"	"
3	5	Whole broth culture	10.0 ml.	210,000 millions	Nil	Nil	Nil
	6	Heat - killed whole broth culture	"	"	"	"	"

Preliminary experiment. This was carried out on goat No. 1 to find out the degree and type of reaction produced by the inoculation of living organisms into one half of the udder and of a Seitz filtrate into the other half. As a reaction was observed the main experiment was begun.

Main experiment. Goats Nos. 2, 3, 4, 7 and 9 were inoculated into one half of the udder with living organisms, dead organisms, or, in one case, with Seitz EK filtrate.

Confirmatory experiment. The reactions of goats Nos. 5 and 6 inoculated in this experiment confirmed the reactions to living or dead organisms noted in the main experiment.

FIG. 1.

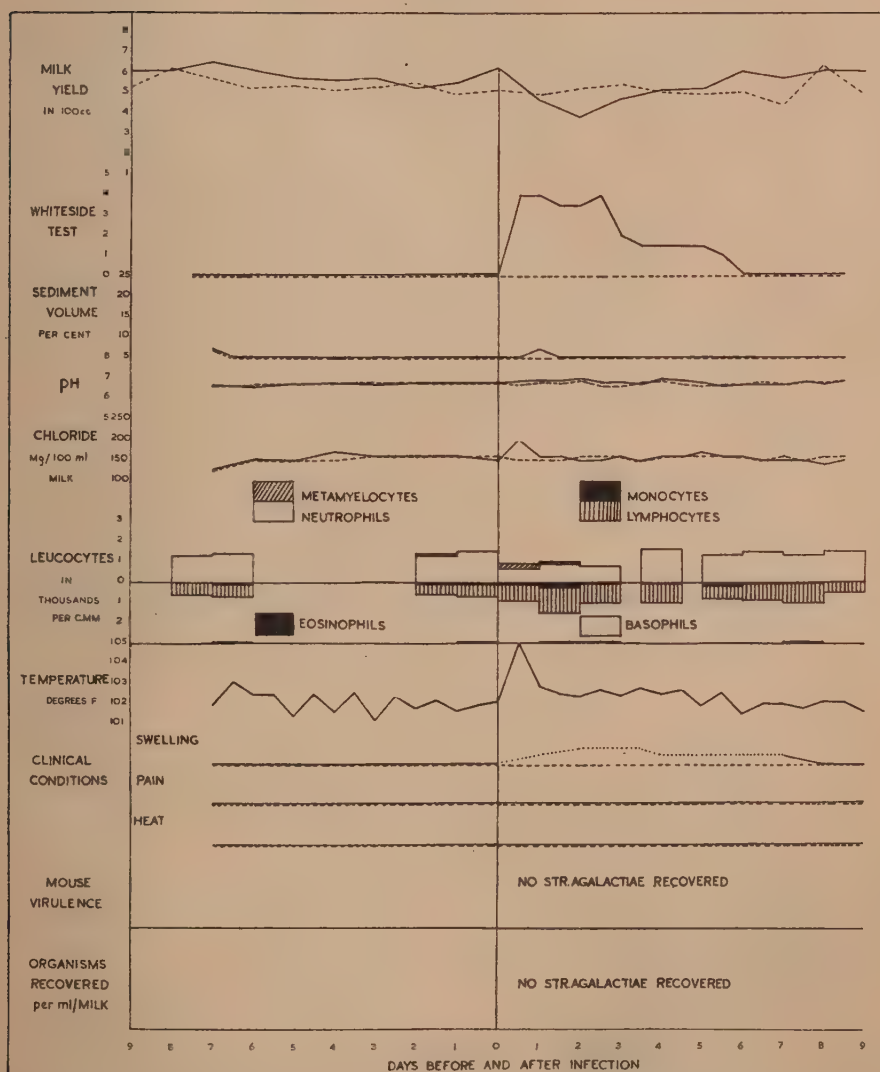


Reactions of goat No. 9 to inoculation into the teat canal of 10 ml. of Seitz EK filtrate, of whole broth culture of *Str. agalactiae* strain S 13.

Whole line - inoculated gland.

Broken line - uninoculated gland.

FIG. 2.

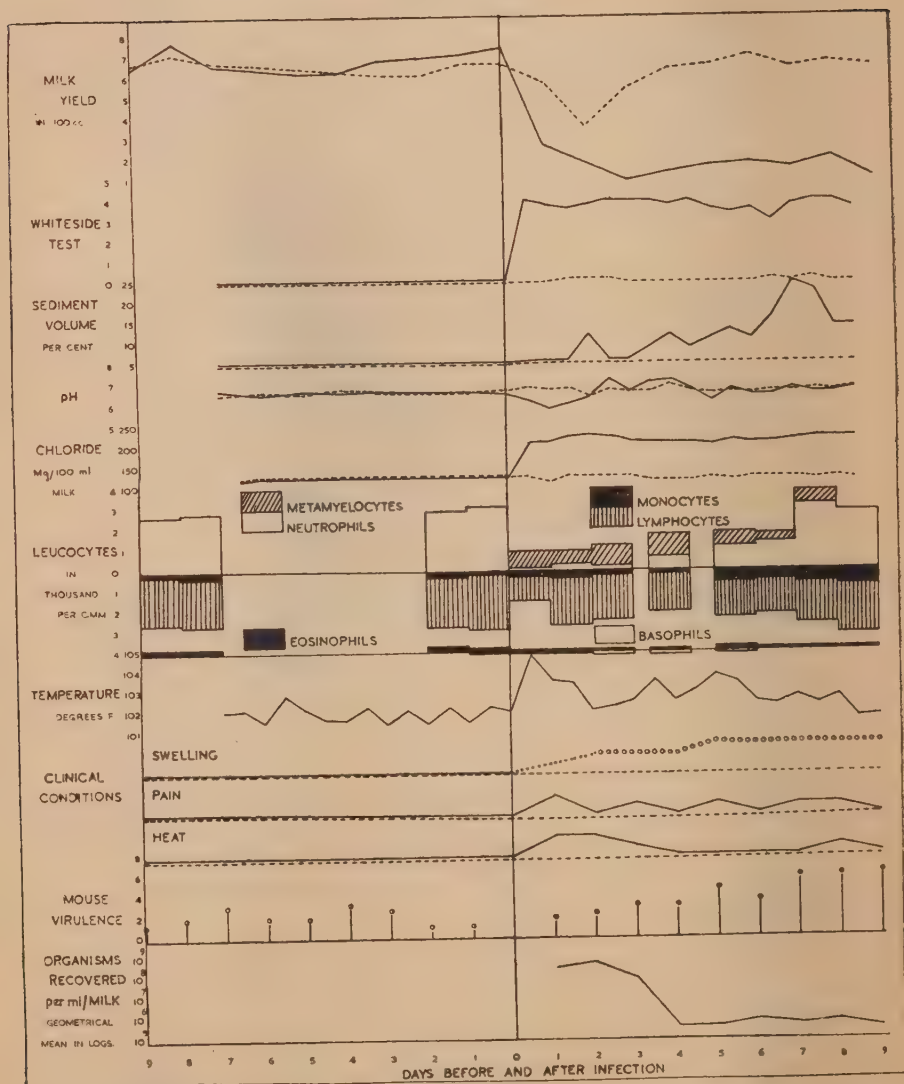


Average reactions of goats Nos. 6 and 7 to inoculation into the teat canal of approximately 100,000 million heat-killed *Str. agalactiae* strain S 13 contained in a volume of 10 ml. of original broth.

Whole line - inoculated gland.

Broken line - uninoculated gland.

FIG. 3.



Average reactions of goats Nos. 1, 2, 3, 4 and 5 to inoculation into the teat canal of approximately 100,000 million living *Str. agalactiae* strain S 13 contained in a volume of 10 ml. of original broth.

Whole line - inoculated gland.

Broken line - uninoculated gland.

RESULTS

Reaction to inoculation of Seitz EK filtrate

No marked reaction occurred (Fig. 1), but such small changes that were recorded were similar in direction to those caused by inoculation of dead or living *Str. agalactiae* (*vide infra*). These changes were—slight rise in temperature, slight drop in milk yield, feeble Whiteside reaction, and a small drop in leucocyte count. No single reaction was in itself significant, but combined they suggested some response to inoculation.

Reaction to inoculation of dead organisms

Clinical. There was a temperature reaction during the 24 hours after inoculation, but no marked general systematic disturbance. No abnormality was detected in the udder of one of the two goats, but in the other goat the inoculated gland was somewhat swollen and of rubber-like consistence from the second to the eighth day.

Milk. A small transitory drop in yield was recorded from each inoculated gland. Milk from these glands was strongly Whiteside-positive for five days after inoculation, and then became negative. The day after inoculation there were very slight rises in sediment volume and in chloride percentage from the inoculated glands; no significant change in pH was noted. No abnormality was detected in milk from the uninoculated glands.

Blood. Within six hours of inoculation there was a drop in total leucocyte count due to an absolute decrease in neutrophil leucocytes, and differential counts showed a tendency for replacement of polymorphs by metamyelocytes. The normal blood picture was restored in one goat on the third day and in the other on the seventh day. For details see Fig. 2 and Table III.

Reaction to inoculation of living organisms

The average of the figures for the five goats (including goat No. 1) is given in Fig. 3, and complete results for one of the animals are shown in Table IV.

Clinical. All goats reacted sharply with a rise of temperature up to four degrees above normal within the first 24 hours and a general systemic disturbance that continued about two days; clinical abnormalities during this period varied from animal to animal and included dullness, inappetence, yawning, grinding of teeth, hiccups, trembling and slight hyperaesthesia. Severity of symptoms varied, but all animals were bright and feeding again by the end of the third day. In two cases the temperature returned to normal after about two days and remained thus until the end of the experiment; in the other three cases the temperature fluctuated between normal and four degrees above normal throughout the experiment. In all animals there was some pain and slight heat in the inoculated gland which lasted a minimum of two days and in one case continued to the end of the experiment. Soft swelling

of the inoculated side of all udders was present in all cases from about the second day after inoculation. In three cases this swelling became progressively harder up to the end of the experiment, while in the other two it became harder for two or three days and then softer again. No abnormality was noted in the uninoculated side of any udder.

Milk. There was a sharp drop in milk yield from both inoculated and uninoculated sides of all udders, but whereas the drop in yield from the inoculated side was maintained throughout the experimental period in the case of four of the five animals, the yield from the uninoculated side had returned to normal within a maximum period of four days; in one animal the yield from the inoculated side showed a tendency to return towards normal over the last five days of the experiment. Milk from inoculated glands was strongly

TABLE
REACTIONS OF GOAT NO. 6 TO INOCULATION OF

Day of Experiment	Time of inoculation									
	-3		-2		-1		+1		+2	
	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.
Milk yield (ml.) ...	360	1,060	470	785	485	1,060	235	645	270	705
Whiteside test (units) ...	0	0	0	0	0	0	4	4	3	4
Sediment volume (%)...	0.3	0.3	0.1	0.1	0.1	0.2	0.3	2.0	0.2	0.1
pH ...	6.7		6.6	6.7	6.8	6.9	6.8	6.9	7.2	7.3
Chloride (mg. per 100 ml.) ...	175	202	175	175	175	165	214	163	163	148
Leucocyte count (10 ³ per c.mm.) ...		5.0		4.7		6.3	3.3*	5.4		8.3
Metamyelocytes (%) ...		0		1		0	11	25		8
Neutrophil adults (%)...	61		63		62		2	17		24
Monocytes (%) ...	11		11		8		17	13		19
Lymphocytes (%) ...	27		23		24		65	43		43
Eosinophils (%) ...		1		1		5	3	1		4
Basophils (%) ...		0		1		1	2	1		2
Erythrocyte count (10 ⁶ per c.mm.) ...		16.0		14.4						
Haemoglobin (g. per 100 ml.) ...		10.5		10.5				11.0		11.0
Packed cell volume (%)		27		25				28		29
Comparative fragility (%) ...		89		97				80		61
Alcohol turbidity (mg. protein per 100 ml.)...		10		13				65		62
Sedimentation rate in saline (mm. per hr.)		2		1				1		2
Specific gravity of plasma		1.03		1.03				1.03		1.04
Temperature ...	101.7	102.2	101.4	102.1	102.4		106.4	102.8	102.4	102.2
Systemic reaction ...				None						
Local reaction of udder				None						

* Six hours

Whiteside positive in all animals throughout the experiment, whereas milk from uninoculated glands was uniformly Whiteside negative.

Sediment volume readings for milk from inoculated glands were abnormal in all goats throughout the experiment, but the degree of abnormality varied considerably in different animals; in general, there appeared to be a positive association between percentage sediment volume determinations and the local reaction of the udder tissue detected clinically. There was no significant alteration in sediment volume readings for milk from uninoculated glands.

The reaction of milk from inoculated glands fluctuated between a pH of 5.4 and 7.8, and remained variable throughout the experiment. By contrast, milk from uninoculated glands remained at a preinoculation level of about pH 6.8.

The chloride percentage of milk from inoculated glands rose

III

HEAT-KILLED *Str. agalactiae* INTO THE TEAT CANAL

+3		+4		+5		+6		+7		+8		+9
p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.
380	810	375	915	375	1,120	405	855	340	930	435	950	410
0	0	0	0	0	0	0	0	0	0	0	0	0
0.1	0.4	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.1	0.1
6.8	6.5	6.9	6.8	6.9	6.7	6.8	6.7	6.8	6.7	6.6	6.8	6.8
148	158	158	170	175	170	165	170	163	158	163	163	163
	4.5				7.0		5.1		4.9		5.1	
	0				0		1		0		1	
	42				63		61		62		54	
	7				10		12		9		8	
	45				25		25		29		36	
	6				1		1		0		1	
	0				1		0		0		0	
											15.5	
	10.5				11.0		12.0		10.5		10.5	
	28				27		30		25		25	
	96				93		85		94		86	
	11				10		22		6		10	
	2				2		2		2		1	
	1.03				1.03		1.03		1.03		1.03	
103.0	102.0	102.8	102.8	102.8	101.4	102.5	100.9	101.8	102.0	101.4	102.2	102.1

Hyperpyrexia. No other abnormality.

Some swelling and rubber-like consistence from 2nd to 8th days.

after inoculation.

during the first 24 hours and remained high in all animals during the first four days; in two goats it returned to within the normal range, but in the other three goats it continued at a high level until the end of the experiment. Milk from uninoculated glands showed no significant alteration.

Str. agalactiae was isolated in large numbers from all milk samples from inoculated glands, but could not be recovered from uninoculated glands.

The geometric mean was used in recording the counts in Fig. 3 in order to modify the high figures obtained on some occasions, particularly in the case of goat No. 5 (Table IV). It can be seen

TABLE
REACTIONS OF GOAT NO. 5 TO INOCULATION OF

Day of Experiment	Time of inoculation									
	-3		-2		-1		+1		+2	
	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.
Milk yield (ml.) ...	400	1,010	445	1,180	395	1,144	130	180	140	310
Whiteside test (units)...		0		0		0	4	3	3	3
Sediment deposit (%)...		0.4		0.1		0.1	2.6	8.4	10.0	22.0
pH ...		6.7		6.8		6.9	5.5	5.2	5.7	5.9
Chloride (mg. per 100 ml.) ...		187		183		183	253	224	236	265
Leucocyte count) 10^3 per c.mm.) ...		5.1		5.0		5.0	2.7*	4.1		3.3
Metamyelocytes (%) ...		0		0		1	14	26		13
Neutrophil adults (%)...		53		57		55	13	4		1
Monocytes (%) ...		9		9		7	6	20		17
Lymphocytes (%) ...		33		33		37	65	48		64
Eosinophils (%) ...		4		1		0	2	2		4
Basophils (%) ...		1		0		0	0	0		1
Erythrocyte count (10^6 per c.mm.) ...		13.0		14.4						
Haemoglobin (g. per 100 ml.) ...		11.0		11.5				11.0		9.5
Packed cell volume (%)		28		25				28		28
Comparative fragility (%) ...		85		93				27		92
Alcohol turbidity (mg. protein per 100 ml.)...		25		18				22		24
Sedimentation rate in saline (mm. per hr.)...		2		1				1		1
Specific gravity of plasma		1.03		1.03				1.03		1.04
Streps. per ml. milk (millions) ...		0		0		0	400	1,000	1,150	900
Temperature ...		102.2	102.9	101.2	102.2	100.2	106.2	103.4	103.4	100.9
Systemic reaction ...			None					Dull, grinding teeth, lack of appetite (2 days)		
Local reaction of udder			None					Hot and tender (2 days)		

* Six hours

from Fig. 3 that the count was in the order of 100 million per ml. for three days and thereafter fell away quite sharply to level out at approximately 100,000 per ml. over the last five days of the experiment. An estimation of the number of streptococci recovered from the milk of each goat over the experimental period vastly exceeded the number inoculated, indicating active multiplication within the udder.

Blood. The outstanding change was a rapid fall in total leucocyte count due to an absolute decrease in neutrophil leucocytes within six hours of inoculation and reaching its maximum about two days later. During this period of neutropenia adult polymorphs were

IV LIVING *Str. agalactiae* INTO THE TEAT CANAL

+3		+4		+5		+6		+7		+8		+9
p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.
145	440	140	640	230	400	200	550	250	310	240	200	210
4	4	4	4	4	3	2	3	3	4	4	4	4
8.4	7.2	7.2	8.0	15.6	6.0	17.2	15.2	13.6	13.6	17.6	20.0	19.2
6.6	6.9	7.0	7.0	6.9	5.3	5.7	5.7	6.3	5.5	5.6	5.7	6.2
274	224	214	195	200	274	282	270	289	289	282	306	321
	5.3				6.1		4.6		3.1		4.6	
	30				48		43		53		41	
	10				15		8		8		12	
	10				7		11		5		7	
	44				28		36		33		39	
	6				1		2		1		1	
	0				1		0		0		0	
											13.1	
	8.0				9.0		9.5		9.5		9.0	
	25				23		27		26		26	
	119				100		90		100		83	
	25				10		11		8		30	
	1				2		1		1		2	
	1.03				1.03		1.03		1.03		1.03	
600	30	0.5	7	150	350	600	1,100	750	200	850	23	96
102.1	102.4	102.8	101.8	102.1	106.2	102.8	103.2	102.5	104.2	102.5	104.9	102.5
Improved (1 day)				Animal bright and eating normally (6 days)								
Slight swelling and some induration (2 days)				Induration progressively more marked (5 days)								

after inoculation.

replaced by immature forms, and at the height of the reaction neutrophils were represented almost entirely by young and band forms. From the third to eighth days of the experiment there was a steady return to pre-inoculation white counts in all animals and counts within the normal range were recorded on the last day.

A slight oligocythaemia was recorded in all animals from the first to eighth days, but had virtually disappeared on the last day. Estimations of coagulation time were restricted to one goat and no abnormality was recorded.

Post-mortem. Bacteriological examination of lungs, heart, liver, spleen and kidneys was negative. The udders were fixed for histological examination immediately after death and no bacteriological examination was possible.

THE INOCULATION OF A LARGE DOSE OF *Str. agalactiae* INTO THE TEAT CANAL OF GOATS CARRYING HAEMOLYTIC STAPHYLOCOCCI

Routine bacteriological examinations of milk samples showed that three animals were regularly excreting haemolytic staphylococci from one half of the udder and the opportunity was taken to determine whether their reaction to a large dose of strain S 13 streptococci differed from that of the other goats whose milk was consistently negative to bacteriological examination. For this purpose two of the animals carrying staphylococci were used. The milk containing staphylococci obtained from these two animals showed a weak irregular Whiteside reaction and slightly abnormal sediment volume. The test doses, prepared as previously described, were inoculated into the uninfected sides; one goat receiving about 81,000 million living organisms and the other the same number of heat-killed organisms.

The reaction to dead organisms was similar to that in goats free from staphylococcal infection, and there was no alteration in the clinical condition of the uninoculated half of the udder from which staphylococci continued to be excreted.

The reaction of the animal inoculated with living organisms was very severe. There was a sharp systemic reaction lasting about two days, with the temperature reaching 107° F. The clinical condition then improved, the animal began to eat, and milk yield from the uninoculated half of the udder rose to within 100 ml. of the pre-inoculation normal.

On the third day after inoculation there was a dramatic change in the clinical picture. The temperature rose again and remained between 105° F. and 107° F. and the animal was dull, slightly stiff about the hindquarters, grinding its teeth, and shivering, and there were occasional spasms of dry coughing. The next day the general condition was worse; the animal was shaking, grinding its teeth, yawning, the ears and head were down, the back was arched, and there was a slight nasal discharge; there was definite induration of both halves of the udder and the uninoculated half (*i.e.*, the half

carrying staphylococci) was very tense. A day later the general condition remained unchanged except that the animal was inclined to lie rather than to stand; the uninoculated half of the udder was now hard and hot, and the inoculated side, although still somewhat indurated, was small by comparison.

On the sixth day the general condition remained unchanged, but the uninoculated side of the udder was now extremely hard and painful and the animal was kneeling on its fore-legs to ease the discomfort caused by the local reaction in the udder. On the following day the only obvious clinical changes were a sunken appearance of the eyeballs and a generally thinner and more angular look due to a general loss of body fat. For humanitarian reasons the goat was destroyed on this day.

The staphylococci disappeared during the first severe systemic reaction and then reappeared in greater numbers to give a local and general clinical picture of severe, unilateral, staphylococcal mastitis. The time of reappearance of staphylococci in milk samples coincided with strongly positive Whiteside readings and increased sediment volume and chloride percentages for these samples.

The blood picture of the goat that developed staphylococcal mastitis differed from that of those that developed streptococcal mastitis in that the initial neutropenia was followed by a continued low count and the presence of a high proportion of immature neutrophils until the day of destruction. Also, lowered determinations for red cell count, haemoglobin, and packed cell volume indicated a severe oligocythaemia.

MOUSE-LETHAL POWER OF *Str. agalactiae* RECOVERED FROM THE MILK OF INOCULATED GOATS

During these experiments in goats the mouse-lethal power of all strains of streptococci recovered from milk samples was compared with that of strain S 13 as originally inoculated. This was done by means of passive protection tests with strain S 13 immune serum prepared in goats. If 0.5 ml. of this serum was inoculated intraperitoneally 18 hours before an intraperitoneal test dose consisting of 0.5 ml. of an 18-hour Todd-Hewitt broth culture of unpassaged strain S 13, it protected some 80 per cent. of mice. The poorest protection afforded by this serum in any one of 23 tests made before goat-passaged strains were examined was 50 per cent., and the best 100 per cent. Results of these 23 tests were:—

	No. of tests	No. of mice inoculated	No. of deaths in 24 hours	Per cent. death rate
Immune serum	23	460	91	20
Non-immune control serum	23	230	222	97

Sample results of passive protection tests with streptococci isolated from one goat are given in Table V and summarised results for all goats are given in Table VI. From the latter table it will be seen that 31 of the 83 strains recovered from milk showed an increase in mouse-lethal power and that there was a statistically significant tendency for the strains of greater lethal power to be isolated late rather than early in the nine-day experimental period.

As an additional test for increased killing power for mice, four strains from three different goats were compared directly in increas-

TABLE V
REACTIONS OF MICE TO S13 BEFORE AND AFTER PASSAGE IN GOATS

<i>Strain S13 isolated from milk on day of experiment No.</i>	<i>Serum</i>	<i>No. of mice inoculated</i>	<i>Died in 24 hours</i>
(Culture as used for inoculation)	Immune	10	2
	Non-immune	5	5
1 (p.m.)	Immune	10	3
	Non-immune	5	5
2 (a.m.)	Immune	10	7
	Non-immune	5	4
2 (p.m.)	Immune	10	3
	Non-immune	5	4
3 (a.m.)	Immune	10	4
	Non-immune	5	5
3 (p.m.)	Immune	10	0
	Non-immune	5	4
4 (a.m.)	Immune	10	1
	Non-immune	5	5
4 (p.m.)	Immune	10	5
	Non-immune	5	5
5 (a.m.)	Immune	10	10
	Non-immune	5	4
5 (p.m.)	Immune	10	10
	Non-immune	5	5
6 (a.m.)	Immune	10	6
	Non-immune	5	5
6 (p.m.)	Immune	10	10
	Non-immune	5	5
7 (a.m.)	Immune	10	7
	Non-immune	5	5
7 (p.m.)	Immune	10	10
	Non-immune	5	5
8 (a.m.)	Immune	10	10
	Non-immune	5	4
8 (p.m.)	Immune	10	9
	Non-immune	5	5
9 (a.m.)	Immune	10	10
	Non-immune	5	5
9 (p.m.)	Immune	10	9
	Non-immune	5	5

The above table shows the results of intraperitoneal inoculation with 0.5 ml. 18-hour Todd-Hewitt broth culture containing not less than 100 million viable organisms per ml. of strain S13 before and after growth in the udder of goat No. 5, of (a) mice previously inoculated intraperitoneally with 0.5 ml. S13 immune serum, (b) mice previously inoculated intraperitoneally with 0.5 ml. non-immune control serum.

TABLE VI
ALTERATION IN LETHAL POWER OF S13

Goat No.	No. of strains examined : isolated on days of experiment Nos.				No. of strains of enhanced lethal power : isolated on days of experiment Nos.			
	1-3	4-6	7-9	Total	1-3	4-6	7-9	Total
1	6	6	5	17	1	1	5	7
2	4	5	6	15	0	5	5	10
3	5	6	6	17	0	1	0	1
4	5	6	6	17	0	0	2	2
5	5	6	6	17	1	4	6	11
Totals	25	29	29	83	2	11	18	31

The above table shows the results of examination of strain S13 for alteration in lethal power for mice after growth in the udder of goats. A strain of enhanced lethal power has been taken as one against which serum protection was less than 50 per cent., this protection being the poorest afforded against strain S13 before passage in any one of 23 serum protection tests.

ing dilution against unpassed strain S 13. Results are given in Table VII, from which it will be seen that one strain (S13/Mi 13) did not exhibit enhanced lethal power, that two strains (S13/H 9 and S13/H 18) showed somewhat greater killing power, and that one strain (S13/C 16) was definitely more lethal for mice than strain S 13 as originally inoculated. Statistical examination of the results for these four strains taken as a group shows a highly significant increase in mouse-lethal power after passage through the udder of the goat (chi squared = 19.63 : p = less than 0.001).

TABLE VII
INOCULATION OF INCREASING DOSES OF S13 IN MICE

Test	Strain	Origin of strain	Strain isolated on day of experiment No.	No. of mice inoculated at each dilution	Died in 24 hours			
					Undiluted	1:8	1:16	1:32
1	S13	As used for inoculation	—	10	9	3	3	1
	S13/H 9	Milk-goat No. 1	9	10	10	9	8	6
	S13/H 18	Milk-goat No. 1	9	10	10	9	7	5
2	S13	As used for inoculation	—	10	9	7	5	3
	S13/C 16	Milk-goat No. 2	8	10	10	10	10	10
	S13/Mi 13	Milk-goat No. 5	6	10	9	7	3	2

The above table shows the results of intraperitoneal inoculation of mice with 0.5 ml. of increasing dilutions of 18-hour Todd-Hewitt broth cultures of (a) strain S13 before growth in udder, (b) strain S13 after growth in udder.

EXAMINATION OF GOAT SERA FOR THE PRESENCE OF ANTISTREPTOCOCCAL ANTIBODIES

Serum samples were taken from all goats before inoculation and again at the end of the nine-day experimental period. Mice were inoculated intraperitoneally with either 1.0 ml. or 2.0 ml. of each of these sera and 18 hours later exposed to an intraperitoneal test dose of 0.5 ml. 18-hour Todd-Hewitt broth culture of unpassaged strain S 13.

Statistical examination of the results of these tests for the goats

TABLE VIII
PROTECTIVE EFFECT OF SERUM FROM GOATS

Goat No.	Inoculum	Serum sample	Dose of serum	No. of mice inoculated	Died within 24 hours
1	Living organisms	Before inoculation	2.0 ml.	10	10
		After "	2.0 "	10	8
2		Before "	1.0 "	10	9
		After "	1.0 "	10	3
		Before "	2.0 "	8	8
		After "	2.0 "	8	3
3		Before "	1.0 "	10	10
		After "	1.0 "	10	6
		Before "	2.0 "	8	8
		After "	2.0 "	8	5
4		Before "	1.0 "	10	8
		After "	1.0 "	10	8
		Before "	2.0 "	8	8
		After "	2.0 "	8	8
5		Before "	2.0 "	10	10
		After "	2.0 "	10	8
6	Dead organisms	Before "	2.0 "	10	10
		After "	2.0 "	10	10
7		Before "	1.0 "	10	10
		After "	1.0 "	10	10
		Before "	2.0 "	8	8
		After "	2.0 "	8	8

The above table shows the results of intraperitoneal inoculation with 0.5 ml. 18-hour Todd-Hewitt broth culture of strain S 13 of mice previously inoculated intraperitoneally with either 1.0 ml. or 2.0 ml. of serum from goats, (a) before inoculation into the teat canal with living or dead strain S 13 streptococci, (b) nine days after inoculation into the teat canal with living or dead strain S 13 streptococci.

inoculated with living organisms (Table VIII) showed a highly significant bias in favour of greater mouse protection by sera obtained at the end of the nine-day experimental period when compared with sera obtained before inoculation ($\chi^2 = 13.7$: $p = \text{less than } 0.001$), although no protective power was demonstrated in goat No. 4. There was no evidence of the development of antistreptococcal antibodies in the sera of the two goats inoculated with dead organisms.

DISCUSSION

The introduction of large numbers of living *Str. agalactiae* produced clinical mastitis in goats and it was possible to measure the extent of the reaction by a variety of tests.

The results show that *Str. agalactiae* multiplied in the udder and that it behaved as a pathogen, for, after the systemic reaction produced by both dead or living organisms had died away, a local reaction persisted. This reaction was manifested by inflammation and was accompanied by changes in the milk that are accepted as the criteria of chronic streptococcal mastitis in cows.

The alteration in mouse-killing power of *Str. agalactiae* after growth in the udder is of considerable interest, especially as the strains of enhanced mouse-killing power were isolated in greatest number from the most severe clinical cases of mastitis. Scrutiny of the complete data available suggests that this increase in mouse-lethal power was the result of the development of virulent variants during growth in the udder, rather than a complete conversion to greater virulence of the whole inoculated strain; thus, single colonies of the strain before inoculation were of standard mouse-killing power, but after growth in the udder occasional colonies of greater mouse-killing power were encountered among colonies of standard killing power. The proportion of colonies of enhanced mouse-killing power appeared to increase when growth in the grossly abnormal udder was prolonged.

The lack of spread of infection from one side of the udder to the other, in spite of the known connection in the blood supply, together with our failure to isolate the organism from any of the major organs of the body, suggests to us that the site of activity of *Str. agalactiae* is outside the general circulation.

The haematological findings are unusual. In previous work (Pattison, 1949a) it had been noted that *Str. agalactiae* inoculated intravenously into mice showed a marked tendency to settle in dense foci in widely separated tissues, and that cellular reaction round these foci was uncommon. If present, the cellular reaction was of a mononuclear type; thus there was no evidence that this organism stimulated the production of leucotaxine.

The inoculation of living streptococci into the udder, however, caused a very definite tissue response: the blood was depleted of neutrophils and there was an intense shift to the left. This mechanism of a severe drop in neutrophil count and shift to the left is unusual and differs from the standard reactions of other diseases; were it not for the continued presence of eosinophils, such a response would be accepted as a fatal neutropenia. It differs from the neutropenia of an anaphylactic reaction because the onset was relatively slow, because it was intransient, and because of the very marked shift to the left; and this marked shift to the left distinguishes it from the standard response to a virus infection. The same blood picture occurred within six hours of inoculation of heat-

killed *Str. agalactiae*, but this returned to normal more rapidly. Injection of Seitz filtrate caused similar changes which were too slight to be called abnormal. This unusual blood picture appears to be a special reaction in which irritation of the udder tissue by certain foreign substances is followed by an escape of neutrophil leucocytes into the milk.

The slight fall in haemoglobin is in accordance with the findings of Robschey-Robbins and Whipple (1936), who found that infections or inflammatory processes inhibit the formation of haemoglobin. This inhibition has been observed by us as an oligocythaemia in other diseases of animals.

The studies recorded here were limited to effects up to nine days after inoculation of large doses of *Str. agalactiae*. In future experiments it is hoped to examine the effects of smaller doses over the same period, and of various doses over longer periods.

SUMMARY

The reactions to inoculation into the teat canal of approximately 100,000 million living *Str. agalactiae* contained in a volume of 10 ml. were a sharp systemic reaction that lasted two to three days, followed by streptococcal mastitis that was clinically, biochemically, and bacteriologically closely similar to bovine streptococcal mastitis.

The reactions to inoculation into the teat canal of approximately 100,000 million heat-killed *Str. agalactiae* contained in a volume of 10 ml. were a brief systemic disturbance, slight abnormalities in the milk, and slight and transitory enlargement of the inoculated half of the udder.

The reactions to inoculation into the teat canal of 10 ml. of Seitz EK filtrate of whole broth culture of *Str. agalactiae* were virtually nil.

The blood picture following inoculation of living *Str. agalactiae* was a severe neutropenia and concomitant shift to the left. The same picture was noted in lesser degree in animals inoculated with heat-killed streptococci.

A goat that was carrying haemolytic staphylococci in one side of the udder developed severe staphylococcal mastitis following inoculation of living *Str. agalactiae* into the opposite side of the udder.

Thirty-one of 83 isolations from milk of the strain of *Str. agalactiae* used for inoculation showed enhanced mouse-killing power after growth in the udder.

Sera obtained from goats nine days after inoculation with living *Str. agalactiae* gave increased mouse protection.

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We wish to express our thanks to our Director, Dr. W. S. Gordon, C.B.E., for his interest in this work, his encouragement, and valued criticism.

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STUDIES OF EXPERIMENTAL STREPTOCOCCAL MASTITIS

II. INOCULATION OF VARYING NUMBERS OF LIVING *Streptococcus agalactiae* INTO THE TEAT CANAL OF GOATS

By

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INTRODUCTION

PATTISON, Taylor and Holman (1950) described an attempt to produce mastitis in goats by injecting large numbers of *Str. agalactiae* into the teat canal. In all cases the injections produced an initial systemic reaction and clinical changes in the udder and milk. Reactions were measured over a period of nine days. Having shown that a large dose could induce reaction in the mammary gland and in order to select a suitable infecting dose for future work, it was desirable to find out whether a smaller dose could produce a similar reaction.

Three experiments were carried out, the first with a group of goats in early lactation, the second with animals in mid-lactation and the third with animals approaching the end of lactation. Six animals were used for each experiment and inoculations were restricted to one half of the udder. The maximum dose was the same as in the previous experiment, *i.e.*, 10^{11} living organisms, and this number was decreased a hundredfold for four succeeding doses to give the series shown in Table I. The sixth goat in each series was given 10^{11} heat-killed organisms. Observations were made for seven days before inoculation and for the succeeding nine days. The goats were then shot and the udders fixed immediately for histological examination.

The methods used and the infecting strain of *Str. agalactiae* selected are described in the paper referred to above. Infecting doses were prepared by growing *Str. agalactiae*, strain S 13, in Todd-Hewitt broth for 18 hours at 37° C. The largest dose was obtained by centrifuging 300 ml. of this broth culture and resuspending the deposit in 10 ml. of supernatant fluid; the smaller doses were made by dilution of culture in normal saline and adjustment to the same volume with the supernatant fluid. The maximum dose in these experiments is referred to in each case as 10^{11} living organisms because the counts fell within the range given by 80 per cent. of 150 previous counts in which the mode was 10^{11} for this strain grown under similar conditions. The actual doses were calculated as 45,000, 92,000 and 210,000 million organisms.

RESULTS

Reaction to Inoculation of Dead Organisms

Control goats reacted similarly to those given the same dose in previous experiments, *i.e.*, there was a brief temperature reaction

after inoculation and a transient drop in milk yield from the inoculated gland. Milk from inoculated glands was Whiteside positive for about five days after inoculation, and showed slightly abnormal sediment volume and chloride percentage values for a short period. Abnormalities in the milk were more marked and more prolonged after inoculation during the later stages of lactation.

Reaction to Inoculation of Living Organisms

Numbers of streptococci isolated per ml. of milk.—Six hours after inoculation the numbers of living *Str. agalactiae* per ml. of milk were similar in all goats, indicating extremely rapid multiplication. Later, in all animals the same type of curve for numbers of living organisms per ml. of milk was observed; high counts were maintained for some 48 hours after inoculation and the numbers thereafter fell sharply to a level of about two million organisms per ml. Table I shows that counts were unrelated to the number of organ-

TABLE I

NUMBERS OF LIVING STREPTOCOCCI PER ML. OF MILK ISOLATED FROM 18 GOATS INOCULATED INTO THE TEAT CANAL WITH VARYING NUMBERS OF *Str. agalactiae* STRAIN S 13

Group of 3 goats	Number of living streptococci inoculated: millions/10 ml.	Average number (millions/ml.) of living streptococci isolated: time after inoculation				
		6 hours	24 hours	36 hours	48 hours	4 days
1	100,000	500	1,033	1,333	550	2.9
2	1,000	1,317	2,517	1,752	36	2.7
3	10	3,821	103	13	10	2.5
4	0.1	502	1,450	333	85	3.0
5	0.001	1,158	2,368	883	20	0.3
6	100,000 dead	0	0	0	0	0

isms inoculated. It is of interest that in some of the goats the numbers per ml. of milk six hours after inoculation were much greater than are generally encountered in the standard broths used for the culture of *Str. agalactiae*. They also greatly exceeded counts recorded during the occasional transitory rises that occurred later in some goats.

Clinical.—No definite correlation was noted between the duration and severity of the systemic reaction and the numbers of organisms inoculated, although in two of the three experiments the most severe reactions were observed in the animals that received large numbers of living organisms; in the third experiment the animals given the two smallest doses reacted severely to the inoculum. In Table II the 15 animals inoculated with living organisms have been grouped according to the type and severity of the local inflammatory reaction of the inoculated gland. This differentiation into several clinical groups emphasises the lack of correlation between the

severity and extent of the inflammatory process on the one hand, and the number of living organisms inoculated on the other.

In our previous paper an account was given of the symptoms shown by goats during the period of general systematic reaction following intramammary inoculation. The same symptoms were observed in these experiments, and, in addition, five of the 15 animals went lame for periods varying from 12 to 48 hours.

Milk.—The sharp drop in milk yield noted in previous experiments was again recorded in every animal, irrespective of the number of organisms injected, and in all animals the yield from the inoculated gland remained well below the normal range throughout the experiment; the yield from the uninoculated gland in each animal

TABLE II

CLASSIFICATION OF 15 GOATS, INOCULATED INTO THE TEAT CANAL WITH VARYING NUMBERS OF LIVING *Str. agalactiae* STRAIN S 13, INTO GROUPS ACCORDING TO THE TYPE OF LOCAL REACTION OF THE INOCULATED HALF OF THE UDDER

No. of goats in group	No. of living organisms inoculated	Average duration of systemic reaction	Type of local reaction of the udder after 60 hours
2	1 goat— 10^9 1 " — 10^{11}	48 hours	Progressive diffuse induration accompanied by pain and heat
2	1 goat— 10^5 1 " — 10^7	60 hours	Progressive diffuse induration without pain or heat
2	1 goat— 10^3 1 " — 10^9	48 hours	Slight transient diffuse induration without pain or heat: then slight lumpy induration without pain or heat
5	2 goats— 10^3 1 goat— 10^5 1 " — 10^7 1 " — 10^{11}	36 hours	Progressive lumpy induration without pain or heat
4	1 goat— 10^5 1 " — 10^7 1 " — 10^9 1 " — 10^{11}	48 hours	Progressive diffuse induration without pain or heat for about 3 days: then clinical improvement to slight swelling only

steadily reverted to normal after the period of systemic reaction had passed. Irrespective of the number of organisms injected, milk from inoculated glands was strongly Whiteside positive and showed markedly abnormal sediment volume throughout the experiment. An exception was a goat inoculated with 10^3 organisms. In this case there were negative Whiteside readings on the 4th, 5th, and 8th days after inoculation, and abnormal sediment volumes were limited to the 1st and 2nd days; leucopenia in this animal was recorded on the 1st day only.

The pH reaction of milk from inoculated glands varied widely and was not associated with the number of organisms inoculated.

There was no evidence that the extent of increased chlorides in the milk was related to the number of organisms inoculated.

Blood.—In our previous paper it was shown that a high concentration of living *Str. agalactiae* caused a marked and rapid leucopenia. The smaller doses used in this experiment produced similar results, but there was no clear association between the number of living organisms injected and the degree of leucopenia produced; further, the leucopenia produced by 1,000 living organisms was much more pronounced than that produced by 100,000 million dead organisms.

Post-mortem.—Bacteriological examination of lungs, heart, liver, spleen and kidneys was negative in all goats. The udders and supra-mammary lymph glands were fixed for histological examination immediately after death and no bacteriological examination was possible.

Mouse Lethal Power of Str. agalactiae Recovered from the Milk of Inoculated Goats

The method described by Pattison *et al.* (1950) for comparing the mouse lethal power of strains recovered from milk was used.

TABLE III

RESULTS OF EXAMINATION OF STRAIN S 13 FOR ALTERATION IN MOUSE-LETHAL POWER AFTER GROWTH IN THE UDDER OF GOATS

Group no.	No. of living organisms inoculated	No. of goats in group	No. of strains of enhanced lethal power in proportion to number of strains examined, on days of experiment nos.			
			1-3	4-6	7-9	Total
1	10 ¹¹	3	2 : 18	5 : 18	5 : 17	12 : 53
2	10 ⁹	3	3 : 18	4 : 17	3 : 17	10 : 52
3	10 ⁷	3	3 : 18	2 : 16	0 : 15	5 : 49
4	10 ⁶	3	3 : 18	7 : 18	5 : 16	15 : 52
5	10 ³	3	0 : 18	0 : 18	3 : 16	3 : 52
Totals			11 : 90	18 : 87	16 : 81	45 : 258

In all, 258 strains were tested and 45 of these were more lethal for mice than the strain as originally inoculated. Table III shows that there was no relationship between the number of strains of enhanced mouse lethal power in a group and the number of living organisms with which animals in that group were inoculated.

Examination of Sera for the Presence of Antistreptococcal Antibodies

Sera taken from all goats before intramammary inoculation and at the end of the nine-day experimental period were tested, as described previously, for the presence of mouse-protecting antibodies. The test could not be carried out with sera from five of the goats because both before and after inoculation mice were killed by serum

alone. The results of these tests are summarised in Table IV. Mouse-protecting antibodies were present in the serum of only one of the 13 goats tested, the animal inoculated with the largest number of living organisms in the second of the three experiments.

Observation on Leucopenia

These observations are based on experimental work carried out previously, in addition to the results obtained in the three experiments reported in this paper.

TABLE IV

RESULTS OF INTRAPERITONEAL INOCULATION WITH 0.5 ML. 18-HOUR TODD-HEWITT BROTH CULTURE OF STRAIN S 13 OF MICE PREVIOUSLY INOCULATED INTRAPERITONEALLY WITH 2.0 ML. OF SERUM FROM GOATS, (a) BEFORE INOCULATION INTO THE TEAT CANAL WITH LIVING OR DEAD STRAIN S 13 STREPTOCOCCI, (b) NINE DAYS AFTER INOCULATION INTO THE TEAT CANAL WITH LIVING OR DEAD STRAIN S 13 STREPTOCOCCI

<i>Group</i>	<i>No. of goats in groups</i>	<i>Serum sample</i>	<i>No. of mice inoculated</i>	<i>Died within 24 hours</i>
Goats inoculated with living streptococci and in which no antistreptococcal antibodies were detected	10	Before inoculation	100	94
		After "	99	88
Goats inoculated with dead streptococci and in which no antistreptococcal antibodies were detected	2	Before inoculation	20	20
		After "	20	20
Goat inoculated with living streptococci and in which antistreptococcal antibodies were detected	1	Before inoculation	6	5
		After "	10	0

The extent of leucopenia recorded in all experiments carried out to date is shown in Table V from which it will be seen that six hours after inoculation the extent of the leucopenia in all animals inoculated with living organisms was much more marked than in all animals inoculated either with Seitz EK filtrate or with heat-killed organisms. Of these 28 goats, 24 were bled at the same intervals after inoculation. Sixteen of these 24 received living organisms, five received heat-killed organisms and three received Seitz EK filtrate. The total leucocyte counts for these animals in their different groups were plotted as shown in Figure 1 and the result emphasises the difference in degree of the leucopenia in the group receiving living organisms as compared with the other two groups. A straight line calculated by the method of least squares has been superimposed over the free-hand line joining counts for the animals inoculated with living organisms, and shows a steady fall of 692 leucocytes per c.mm. per hour.

Observation on Accidental Infection with Str. agalactiae Strain S 13

During previous experiments on the intramammary inoculation of goats with *Str. agalactiae* strain S 13, two animals were accidentally infected in circumstances that suggested that only a very small number of living streptococci of this strain was required to cause infection. These two cases are briefly reported because they differed in one very significant respect from intentional infections. The two animals were inoculated with 10 ml. of the same Seitz EK

TABLE V

THE LEUCOPENIA PRODUCED SIX HOURS AFTER INOCULATION INTO THE TEAT CANAL OF LIVING OR HEAT-KILLED *Str. agalactiae*, OR OF SEITZ EK FILTRATE OF WHOLE BROTH CULTURE

Inoculum	Number of goats in group	Average leucocyte count thousands/c.mm.		Highest count in group at 6 hours
		Before inoculation	6 hours after inoculation	
Seitz EK filtrate ...	3	6.3	5.4	6.2
10 ¹¹ heat-killed organisms...	6	8.3	5.1	9.8
10 ⁸ living organisms ...	3	6.2	3.2	4.5
10 ⁶ " " ...	3	6.0	2.9	4.3
10 ⁷ " " ...	3	7.5	3.4	3.7
10 ⁹ " " ...	3	6.4	2.8	3.0
10 ¹¹ " " ...	7	7.1	2.6	3.8

filtrate of an 18-hour Todd-Hewitt broth culture of strain S 13. Neither goat reacted immediately to inoculation, but at 24 hours one had a temperature of 104.8 and was dull and listless. The reaction in the other animal was delayed until three days after inoculation when a temperature of 104.8 was recorded. At the time of the systemic reaction both animals showed high counts of *Str. agalactiae* in milk from the inoculated glands only.

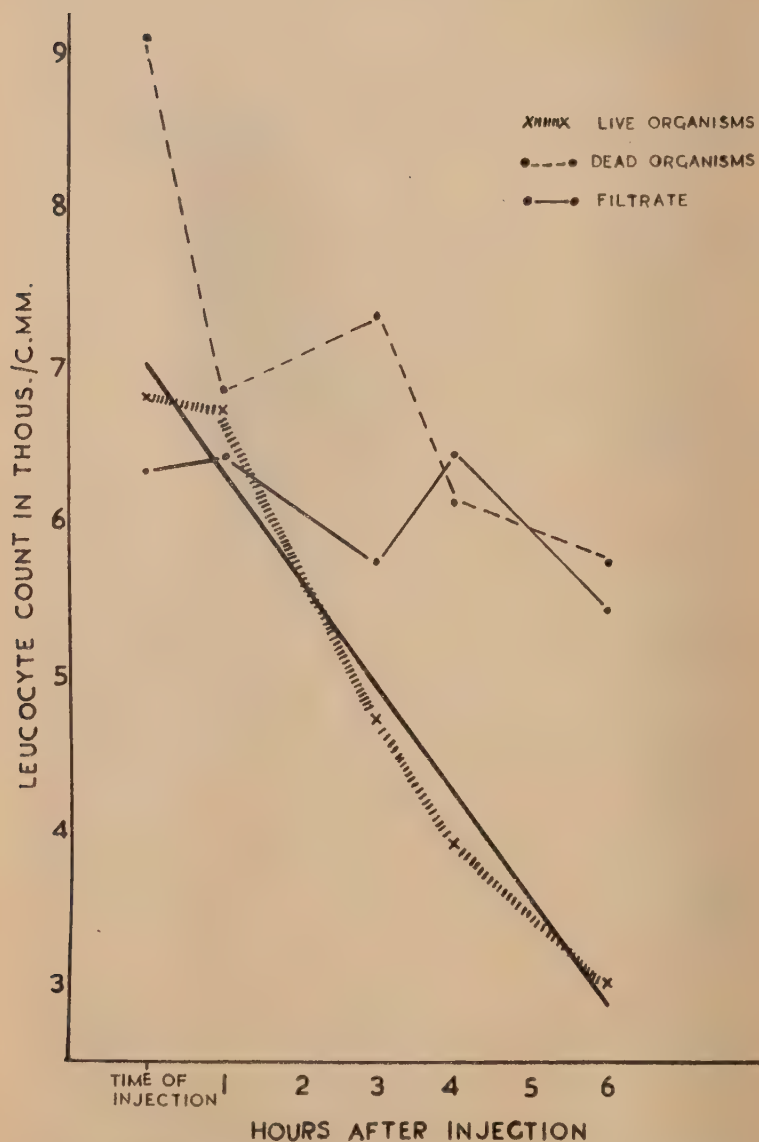
As these were the only two cases in which the systemic reaction was not obvious six hours after inoculation and because a plate count made on the filtrate at the time of inoculation was negative, the presumption is that only a very few streptococci had passed through the filter and that the systemic reaction was in consequence delayed.

DISCUSSION

The results of the experiments reported in this paper show that the disease produced by the various doses used bore no relationship to the number of living organisms inoculated. The volume of inoculum used was the same in every case (*i.e.*, 10 ml.) and it is possible that this may be of importance in creating the disease. It cannot safely be assumed that 1,000 living organisms would necessarily have the same effect in a greatly reduced volume.

In previous work with mice the lethal power of strain S 13 was found to be closely related to the number of living organisms in-

FIG. 1



Showing fall in leucocyte count over first six hours. The solid line passing through the points given for live organisms is based on the method of least squares.

oculated, irrespective of the volume of inoculum used (Pattison, 1949). This is in striking contrast to the results obtained in the experiments described above since here there was no obvious relationship between the number of living organisms inoculated and the effect produced. The steady fall in leucocyte count during the first six hours, which bore no relation to the numbers of living organisms inoculated, suggests a mechanical leakage of leucocytes from blood to milk, rather than the changes usually associated with inflammation. The presence of immature neutrophils in the peripheral blood suggests that if neutrophil cells fall below a certain threshold, immature forms are allowed to escape into the circulation.

The fact that a dose calculated as 1,000 living organisms in 10 ml. was as effective in producing disease as a dose, in the same volume, 100 million times as great, suggests that a relatively small number of living streptococci S 13 can be relied upon to cause disease when inoculated in the manner described. This inference is supported by the infection that followed the introduction into the teat canal of an imperfect filtrate containing what was presumed to be an extremely small number of living organisms. The standardisation by dilution of very small doses of living organisms becomes increasingly difficult as dilution proceeds and for this reason it is suggested that a dose of 1,000 living streptococci in 10 ml. might be taken as an effective infecting dose.

SUMMARY

The inoculation of doses of living *Str. agalactiae* varying from approximately 1,000 to 100,000 million and contained in a volume of 10 ml. into the teat canal of goats produced a sharp systemic reaction that lasted two or three days, followed by a streptococcal mastitis that was clinically, biochemically and bacteriologically closely similar to bovine streptococcal mastitis. The degree and type of reaction could not be correlated with the number of living organisms inoculated.

An examination of the infecting strain of *Str. agalactiae* showed that 45 out of 258 isolations from milk were more lethal for mice after growth in the udder.

The serum of one of 13 goats tested nine days after inoculation with *Str. agalactiae* gave increased mouse protection.

During the first six hours the leucocyte counts of goats inoculated with living organisms fell as an arithmetical progression.

The onset of the systemic reaction was delayed in two goats accidentally infected with what were believed to be very small numbers of living streptococci.

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STUDIES ON EXPERIMENTAL STREPTOCOCCAL MASTITIS

III. OBSERVATIONS ON AN ALTERATION IN MOUSE-LETHAL POWER OF *Streptococcus agalactiae*, STRAIN S 13 DURING GROWTH IN THE UDDER OF GOATS

By

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INTRODUCTION

In two previous papers (Pattison, Taylor and Holman, 1950*a* and 1950*b*) we described the reaction that followed the inoculation of *Str. agalactiae* strain S 13 into the teat canal of lactating goats. Over a period of nine days, all strains of this organism isolated from milk were examined and 76 of 341 showed enhanced mouse-killing power. Further examination of the data summarised in these papers suggested that the strains of enhanced mouse-killing power were not haphazard in occurrence but were related to an increase in body temperature and to a relatively high count of viable organisms in the milk at the time of isolation. Evidence on which this conclusion is based is recorded here.

MATERIALS AND METHODS

The general methods used have been described in the papers referred to above. The complete data for the determination of the relationship between mouse-lethal power and body temperature were available for 59 strains of enhanced lethal power and 212 strains of standard lethal power, isolated as single colonies from the milk of 19 different goats. Data on the relationship between lethal power and viable count per ml. of milk at the time of isolation were available for 51 strains of enhanced lethal power and 200 strains of standard lethal power, isolated from the milk of 18 different goats.

Method of Preparing Mouse-protecting Sera in Goats

The killing power of a strain for mice was determined by a passive protection test as described briefly in a previous paper (Pattison *et al*, 1950*a*) with strain S 13 immune serum prepared in goats. As the criterion for differentiation between strains of standard and of enhanced mouse-lethal power depended entirely on the result of this test, the methods of preparing the goat-serum and of carrying out the test will be given in detail here.

The goat serum was obtained from one or other of two animals, both of which had been used originally for pathogenicity studies with strain S 13. One animal had received a series of inoculations of living whole broth culture intravenously and the other a similar series of inoculations intraperitoneally; details are given in Table I.

Both goats reacted violently to the initial inoculation and both aborted. Several jugular blood cultures from the animal inoculated intravenously and one from the animal inoculated intraperitoneally were positive for streptococci during the first three days after inoculation, and group-B streptococci were isolated in pure culture from the liver, kidney and pleural fluid of the foetus from the animal inoculated intravenously. The animal inoculated intraperitoneally was clinically normal five days later, but the animal inoculated intravenously went lame in a hind-leg five days after the first inoculation and this lameness has persisted over a period, to date, of 58 weeks.

Mouse protection was not afforded by either serum at a test on the 29th day after inoculation, but on the 36th day both protected

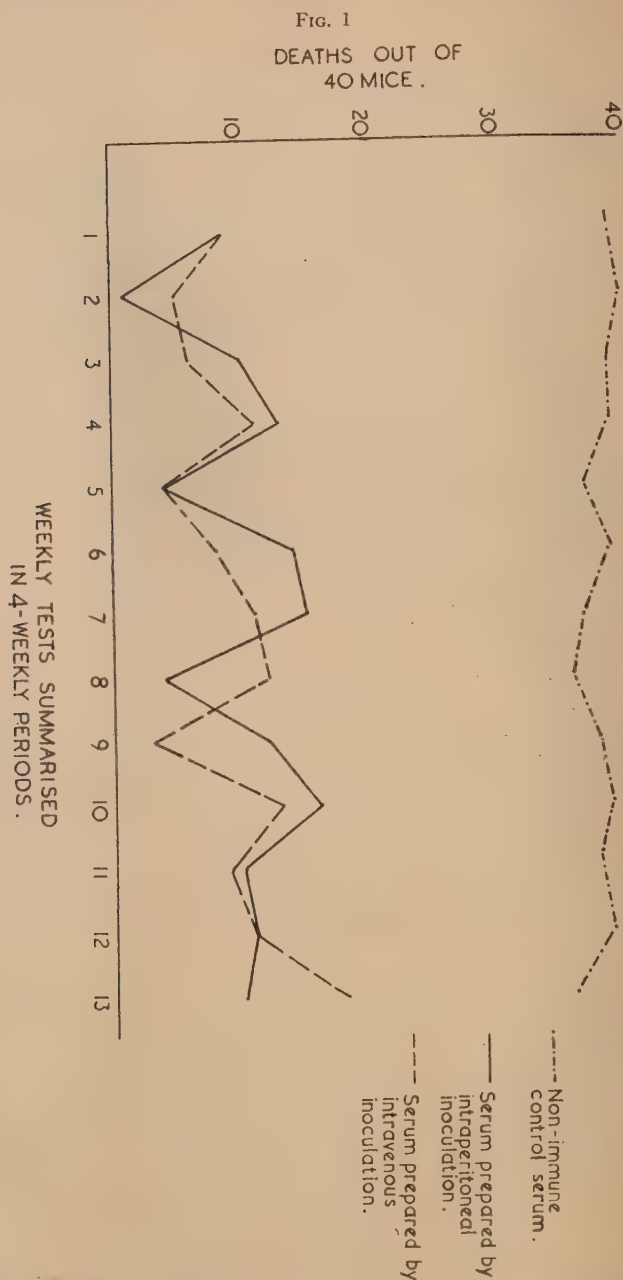
TABLE I
IMMUNISATION OF GOATS USED TO PRODUCE MOUSE PROTECTING SERA

<i>Days from first inoculation</i>		0	13	20	29	30	36	37	43	44	49	51	57	58
Goat inoculated intravenously	Dose of culture (ml.)	50		5		5		5		5		5		5
	Presence or absence of mouse protection	—		—		+		+		+		+		+
Goat inoculated intraperitoneally	Dose of culture (ml.)	50		5		5		5		5		5		5
	Presence or absence of mouse protection	—		—		+		+		+		+		+

Immunising dose: 50 ml. or 5 ml. of strain S 13 in Todd-Hewitt broth to give culture density of at least 100 million viable organisms per ml.

about 90 per cent. of the mice tested. The results of further tests are shown in Fig. 1 and it can be seen that the average mortality using serum from each goat was about 25 per cent. and never exceeded 45 per cent., compared with an average mortality for non-immune sera of 95 per cent. (highest 100 per cent., lowest 90 per cent.) The figure also shows that the mouse-protecting potency of both sera varied little over the 13-month period. All isolations of single colonies of strain S 13 obtained from goats were tested for lethal power against one or other of these two sera.

The production of mouse-protecting sera in the two goats involved inoculations totalling 80 ml. of living whole culture in Todd-Hewitt broth injected either intraperitoneally or intravenously over a period of 58 days. More recent work has shown that similar mouse-protect-



Results of passive mouse protection tests against strain S 13 streptococci, using immune serum prepared in goats either by intravenous or intraperitoneal inoculation of living organisms.

ing titres can be produced with much smaller and fewer doses of living strain S 13 streptococci inoculated intraperitoneally. For example, the sera of two goats given four intraperitoneal inoculations of 0.5 ml., 1.0 ml., 2.0 ml., and 2.0 ml. living whole broth culture over a period of 21 days showed, six days after the final inoculation, a protecting titre of similar potency to that described above. The titre of these sera, when freshly drawn from the goat, has remained unchanged over three months.

Method of Testing Mouse-lethal Power

The method used for testing mouse-lethal power was based on the observation that mouse-protecting antibodies against group-B streptococci could be demonstrated in guinea-pig serum by intraperitoneal inoculation of mice with serum followed, 24 hours later, by an intraperitoneal test dose of living culture (Pattison, 1948). In the present work all tests for mouse-lethal power of strains isolated from goats were carried out by the intraperitoneal inoculation of mice with 0.5 ml. of immune goat serum between 2 p.m. and 4 p.m. Eighteen hours later, 0.5 ml. of an 18-hour whole culture in Todd-Hewitt broth of the streptococcal strain to be tested was injected intraperitoneally. Each test was carried out with 15 mice; ten previously inoculated with immune serum and five with non-immune serum. Strains isolated from the milk of each goat during the nine-day period were examined at the same test and these were compared with strain S 13 as originally used for inoculation of the goat. In most cases the test for mouse-lethal power was carried out within one month of isolation of strains, but in a few cases one sub-culture on bacto-tryptose agar was required to maintain viability until the test could be performed. The immune sera were always used within 48 hours from the time of withdrawal from the goat.

RESULTS

Relationship of Increased Mouse-lethal Power to a Rise in Body Temperature

Inspection of the data showed that any rise in temperature, after the immediate reaction to inoculation (usually lasting about 48 hours) had died away, was likely to be associated with an increase in virulence. This was investigated in two ways.

A. Criterion of Increased Mouse-lethal power.—A strain was considered to show enhanced lethal power if, at the test described above, it killed six or more mice out of the ten inoculated within 24 hours. The lethal power of the original strain in the 19 tests carried out is shown below:—

No. of mice killed in 24 hours	0	1	2	3	4	5
No. of tests	1	5	7	4	0	2

It can be seen from this distribution that the maximum death rate of S 13 did not exceed five mice (50 per cent.).

B. Criterion of An Abnormally High Body Temperature.—

A temperature was taken as being abnormal if it clearly exceeded by a minimum of 0·5 F. the highest temperature recorded at twice-daily readings over a minimum period of seven days before inoculation.

Using these criteria, the following four-cell table was obtained:—

			<i>Abnormal</i> temp.	<i>Normal</i> temp.
Increased mouse lethal power ...	...	...	22	37
Standard mouse lethal power ...	...	...	20	192

These figures gave chi squared as 27·34 and p less than 0·0001. This shows a highly significant relationship between enhanced mouse-killing power and raised body temperature.

Relationship of Increased Mouse-lethal Power to a High Viable Count per ml. of Milk

Enhancement of mouse-lethal power was judged by the same standard as in the foregoing paragraph. The actual counts for the number of organisms in 1 ml. of milk, expressed as powers of 10,

TABLE II

DISTRIBUTION, IN RELATION TO THE VIABLE COUNT PER ML. OF THE MILK SAMPLE FROM WHICH THEY WERE ISOLATED, OF 51 STRAINS OF ENHANCED MOUSE-LETHAL POWER AND 200 STRAINS OF STANDARD MOUSE-LETHAL POWER

Group	Viable count	Number of strains of enhanced lethal power	Number of strains of standard lethal power	Strains showing enhanced lethal power, as percentage of total
1	10 ³	1	21	4·5
2	10 ⁴	4	37	9·7
3	10 ⁵	6	56	9·7
4	10 ⁶	12	54	18·2
5	10 ⁷	10	17	37·0
6	10 ⁸	9	11	45·0
7	10 ⁹	9	4	69·0

were used in determining the relationship of lethal power to count. The distribution of viable counts for 51 strains of enhanced lethal power and 200 strains of standard lethal power given in Table II shows a steady rise in the proportion of strains of enhanced lethal power as the viable count increased. The result is obviously significant with a value for chi squared of 43 for the six degrees of freedom, giving p as much less than 0·0001.

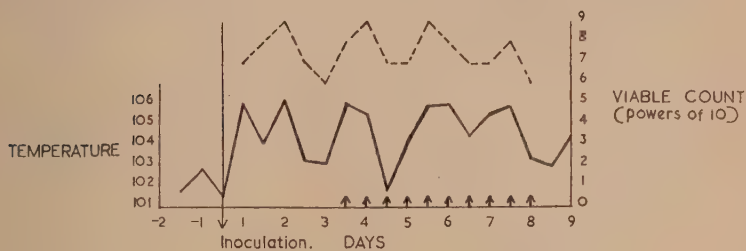
Examples of the relationship of mouse-lethal power of isolations of strain S 13 to raised body temperature and to high viable count per ml. of milk at the time of isolation from three selected goats are depicted in Fig. 2. In the case of goat A, cultures of enhanced killing power were closely related to high body temperature and

high viable count; in goat B, temperature and counts were low and cultures of standard lethal power only were isolated; in goat C, the only two cultures of enhanced lethal power were related to rises in temperature and in viable count within a 12-hour period.

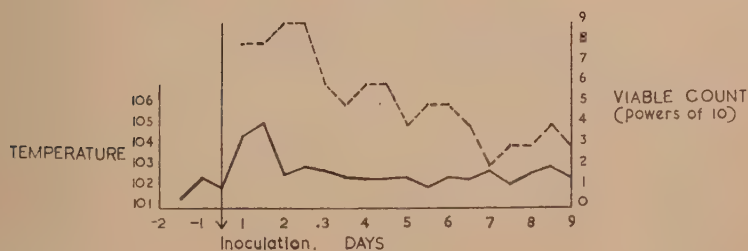
Retention of Mouse-lethal Power after Storage in the Dried State

After the initial test for mouse-lethal power, a 48-hour growth of each strain on bactotryptose agar was rubbed up in sterile rabbit

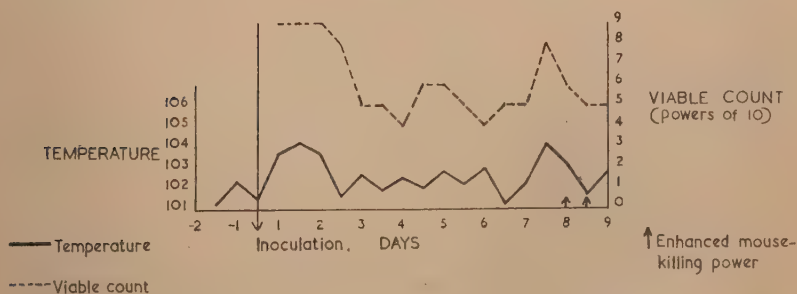
FIG. 2
GOAT A



GOAT B



GOAT C



The relationship between mouse-lethal power, body temperature and viable count per ml. of milk for three selected goats,

serum, dried over phosphorus pentoxide and sealed *in vacuo*. To test whether mouse-lethal power was maintained during preservation in the dried state, a random selection of nine strains of enhanced mouse-lethal power from nine different goats was tested after periods of storage varying from one to seven months. The results before and after drying are given in Table 3. It will be seen that with the exception of strain S 13/K 4 all had retained their enhanced mouse-killing power to a significant extent.

DISCUSSION

The serological type within group-B of strain S 13 was designated Type 1 ? subtype (Pattison, 1948), and it would have been highly desirable to have established that the isolations of enhanced mouse-

TABLE III

RESULTS OF PASSIVE PROTECTION TESTS IN MICE USED TO DEMONSTRATE MOUSE-LETHAL POWER OF ISOLATIONS OF STRAIN S 13 BEFORE AND AFTER STORAGE IN THE DRIED STATE

Strain	Mouse-lethal power when first isolated		Period of storage in the dried state (months)	Mouse-lethal power after storage	
	Deaths in 24 hours out of 5 non-immune mice	Deaths in 24 hours out of 10 immune mice		Deaths in 24 hours out of 5 non-immune mice	Deaths in 24 hours out of 15 immune mice
S 13 (as used for inoculation of goats) ...	5*	2*		5	4
S 13/K4 ...	4	6	1	5	5
S 13/Be 11 ...	5	7	1	5	7
S 13/R 15 ...	5	9	2	5	13
S 13/B 15 ...	5	8	2	5	8
S 13/Chl 15 ...	5	10	5	5	14
S 13/Mi 8 ...	4	10	6	5	13
S 13/Ed 12 ...	5	10	6	5	14
S 13/A 13 ...	4	10	6	5	13
S 13/Cha 9 ...	5	8	7	5	12

* Average for 19 tests.

lethal power of this strain from goats' milk were of the same serological type. This could not be done because no satisfactory type-specific serum for this strain was available. We have no reason to suppose that the strains of enhanced mouse-lethal power differed in type from the parent strain; moreover, the fact that strains different in mouse-lethal power from the parent strain were always *more* and never less lethal indicated that the same sort of variation had occurred in each case. Throughout these experiments special care was taken not to introduce any strain of streptococci other than S 13 into the compound where the goats were housed. In no instance

were streptococci isolated from any goat or any udder not inoculated with strain S 13.

There was a day to day association of mouse-lethal power and body temperature, and of mouse-lethal power and viable count per ml. of milk. The tests were made every 12 hours over the nine-day period. It was found in several instances that, although the rise in variable count or in body temperature did not always coincide with the isolation of a culture of enhanced lethal power, there was a relationship within a 12- to 24-hour period.

The observations discussed in this paper indicate that *Str. agalactiae* strain S 13 was possibly in an unstable condition in the udder of the goat and that its rate of multiplication and lethal power for mice might, in these circumstances, alter from time to time. These facts may be fundamental in explaining the behaviour of *Str. agalactiae* as a pathogen.

SUMMARY

Individual colonies of *Str. agalactiae* strain S 13 were isolated from the milk of goats after the introduction of a culture into the udder.

An enhancement in the lethal effect on mice was related to rises in body temperature and to the viable count per ml. of milk at the time of isolation.

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SOME OBSERVATIONS ON THE STATUS OF MINERAL NUTRITION OF NIGERIAN CATTLE

By

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INTRODUCTION

WITH the effective control of infectious disease in Africa, mineral deficiencies in pasture will become of increasing economic importance. The purpose of this work was to investigate the deficiencies which, in the light of the evidence available, might possibly be encountered in the vicinity of this laboratory on the Nigerian Plateau.

Cattle.—Cattle from three sources were used in the investigation: (a) from the stock maintained at this laboratory for experimental purposes; (b) from the dairy herd at the Livestock Improvement Centre, Vom, and (c), in one case, on loan from a local Fulani herd. The great majority were of the White Fulani type. Wherever possible, wet and dry blood smears were examined daily to ensure the absence of blood parasites and faecal egg counts were carried out weekly.

Normal Feeding Standards of Cattle.—During the wet season lasting approximately from April to September inclusive, laboratory cattle are allowed free range and receive no supplements. For the remainder of the year, when grazing is scarce, they are fed on hay together with a concentrate such as cotton-seed or ground-nut cake, and rock salt when the latter is available.

In the wet season cows from the Livestock Improvement Centre receive, according to age and milk yield, guinea-corn, palm-kernel cake and ground-nut cake to supplement grazing. In the dry season milking cattle are fed on maize, silage and hay with the same concentrates. The usual nomadic Fulani herd is allowed free range throughout the year, receiving no supplementary feeding stuffs other than native salts when these are available, but the Fulani owner of the particular herd in which osteophagia was observed (*vide infra*) fed cotton-seed occasionally.

Pasture.—The commoner grasses found at the Laboratory Vom include *Eragrostis* species, particularly *E. pilosa* and *E. aspera*, *Eleusine indica*, *Dactyloctenium aegypticum*, *Chloris pilosa*, and other *Chlorosis* species, *Cynodon dactylon*, two species of *Microchloa*, *Sporobolus pyramidalis* and other species, *Aristida* species, *Perotis indica*, *Leersia hexandra*, several species of *Loudetia*, *Panicum*, *Setaria* and *Echinochloa*, *Paspalum scorbiculatum* var.

commersonii, *Brachiaria* species, *Digitaria* species, *Tricholaena* species, *Melinis* species, several species of *Pennisetum*, *Imperata cylindrica*, two species of *Sacchaum*, several varieties of *Sorghum* including *S. guineense*, several species of *Andropogon* and *Hyparrhenia*, *Heteropogon contortus*, *Teleopogon elegans* and *Roetboellia exaltata*. The list is by no means complete, many important genera having been omitted.

Some of the grasses listed are valuable fodder plants and include members used by man as a staple food or an occasional food in time of scarcity.

CALCIUM, MAGNESIUM AND PHOSPHORUS

For the sake of convenience these elements will be considered together.

It has long been thought that cattle in the vicinity of the Veterinary Station at Vom might suffer from a phosphorus deficiency, since the soil and pasturage are known to be poor in this element. This contention received added support when it was observed recently that cattle on loan from a local Fulani herd were chewing bones, and also when reports from the Agricultural Department on the results of manurial trials carried out in Vom revealed that both phosphorus and lime were beneficial to the growth of test crops. There was already some reason to believe that a shortage of calcium might exist in the normal diet, as geophagia, which is very commonly seen in Nigerian cattle, can be successfully counteracted by feeding native salts rich in calcium.

At the time this work was commenced there was no suggestion of a deficiency of magnesium and this element was included in the investigation only as a matter of interest. More recently, however, reports have been received that local crops, particularly guinea-corn, may contain suboptimal amounts of magnesium and, as rations deficient in magnesium may produce hypo-magnesaemia, more attention was focused on it.

Calcium, Magnesium and Phosphorus Content of Pasture

The results of analyses of grass samples from 20 areas in the immediate vicinity of Vom and of three non-leguminous hays are given in Table I, which also includes, for purposes of comparison, figures obtained by other workers for grass and hay from different parts of Nigeria, from South Africa and from Europe.

The phosphorus content of the grass, although low as compared with average English pasture grass, compares favourably with that of other Nigerian grasses and is considerably higher than that of the best Veldt grass, particularly when it is considered that the samples were collected over the period October 1948 to January 1949 when the quality was at its lowest. The phosphorus content of the local hay is, however, very low. The calcium and magnesium

content of both grass and hay is low compared with English figures.

The calcium-phosphorus ratios of the grass and hay samples examined were:—

Grass (60 samples). 10·4:1–1:2·3, av 1·5:1.

Hay (three samples). 3·6:1–2·5:1, av. 3·1:1.

With the exception of two individual samples from the same area (Ca: P, 10·4: 1 and 4·3:1) the ratio for grass fell within the range 2:1:1 and 1:2·3.

Calcium, Magnesium and Inorganic Phosphorus Content of Blood

Results obtained with three groups of animals grazing within the environs of Vom are given in Table II, together with figures published by Anderson (1933) for White Fulani cattle in Northern Nigeria, by Jones (1943) for a mixed group of Ankole and Zebu cattle in Uganda and by Allcroft (1947) for English cattle. Blood samples from the Vom animals were obtained during the dry seasons of 1946-47, 1947-48 and 1948-49.

TABLE I
THE CALCIUM, MAGNESIUM AND PHOSPHORUS CONTENT OF NIGERIAN,
S. AFRICAN AND EUROPEAN GRASSES AND HAYS

	Calcium (as CaO) (% dry matter)		Magnesium (as MgO) (% dry matter)		Phosphorus (as P ₂ O ₅) (% dry matter)	
	Range	Mean	Range	Mean	Range	Mean
<i>Grasses</i>						
Pasture grass from Vom area (Garner, 1948)	0·21–0·94	0·37	0·08–0·66	0·23	0·15–0·97	0·44
Pasture from Shika area, N. Nigeria (Anderson, 1933)	0·43–0·89	0·50	—	—	0·14–0·58	0·30
Other N. Nigerian pastures (Anderson, 1933)	0·30–0·91	0·60	—	—	0·20–0·51	0·34
Veldt grasses, S. Africa (Henrici, 1930)	—	—	—	—	0·05–0·16	—
British pasture grass (rota- tional close grazing) (Hal- nan and Garner, 1940)	—	0·28	—	—	—	0·16
British pasture (Godden, 1926)	0·56–1·00*	—	—	—	0·60–0·73*	—
British pasture (Blakemore and Stewart, 1934–35)	—	—	0·61–1·01	0·81	—	—
<i>Hays</i>						
From Vom area (Garner, 1948)	0·08–0·22	0·15	0·04–0·19	0·10	0·03–0·12	0·09
Non-leguminous European hay (Linton, 1931)	0·05–1·15	—	—	—	0·16–1·07	—
Mixed British hay (Linton, 1931)	0·32–2·30	0·99	—	—	0·29–0·75	0·40

* The higher figures refer to the average value for good pasture and the lower figures to that for poor hill pasture.

Group 1 (consisting of 48 animals from the Livestock Improvement Centre and 23 Laboratory cattle; all apparently healthy). Despite the difference in feeding standards of Laboratory and Livestock Improvement Centre cattle, the blood pictures were almost identical, and the results obtained from these animals are therefore presented in one group.

The figures for calcium did not differ widely from Allcroft's, but were significantly lower than those obtained by Jones for similar cattle in East Africa. All values for magnesium fell within the normal range for English cattle and were not significantly different from those obtained with East African Zebus. Inorganic phosphorus levels were higher than those given by Allcroft, but significantly lower than those given by Jones.

Group 2 (17 osteophagic animals from a local Fulani herd). The only significant difference between the figures obtained for this group and for Group 1 was in the blood calcium, which was higher in this group.

Group 3 (30 laboratory animals experimentally infected with *Trypanosoma congolense*). These animals are included since it is well known that the geophagia so often seen in Nigerian cattle is accentuated in trypanosomiasis. The average blood calcium level of this group was significantly lower than that of Group 1. The difference in magnesium levels in Groups 1 and 3 is barely significant.

TABLE II

THE CALCIUM, MAGNESIUM AND INORGANIC PHOSPHORUS CONTENT OF THE BLOOD OF ZEBU CATTLE IN NIGERIA AND UGANDA AND OF ENGLISH CATTLE

	Serum calcium (mg. per 100 ml.)		Serum magnesium (mg. per 100 ml.)		Whole blood inorganic phosphorus (mg. per 100 ml.)	
	Range	Mean	Range	Mean	Range	Mean
Zebu cattle, Nigeria						
*Vom, Group 1	6.4-13.0	10.5 $\pm$ 1.07	1.8-3.1	2.6 $\pm$ 0.30	3.2-9.8	5.1 $\pm$ 0.92
" 2	9.8-13.1	11.4 $\pm$ 0.83	2.0-3.1	2.6 $\pm$ 0.31	4.0-8.4	5.5 $\pm$ 0.98
" 3	7.3-11.7	9.8 $\pm$ 0.93	1.8-3.0	2.4 $\pm$ 0.29	3.2-6.3	5.1 $\pm$ 0.84
Shika (Anderson 1933)	—	11.4	—	—	3.7-6.9	4.8
Zebu cattle, Uganda (Jones, 1943)	9.2-14.5	11.5 $\pm$ 1.38	2.1-3.7	2.8 $\pm$ 0.46	2.8-8.4	5.8 $\pm$ 1.35
English cattle (Allcroft, 1947)	8.5-11.4	10.0 $\dagger$	1.8-3.2	2.4 $\dagger$	3.1-6.0	4.6 $\dagger$

* Group 1.—Healthy cattle from the Livestock Improvement Centre, Vom, and from this laboratory (71 animals).

Group 2.—Animals exhibiting osteophagia (17 animals).

Group 3.—Laboratory animals experimentally infected with *T. congolense* (30 animals).

$\dagger$ Estimated from Allcroft's data.

The question at once arises as to whether the calcium and inorganic phosphorus levels recorded in Group 1 can be considered "normal," for both values are significantly lower than those given by Jones. It is possible that the supply of calcium and phosphorus is adequate to prevent the development of clinically evident deficiencies and yet not optimal. Anderson found that mineral supplements increased the blood inorganic phosphorus of his cattle appreciably. At this laboratory bonemeal supplements produced no elevation of the blood calcium and phosphorus.

On this evidence it may be concluded that the animals in Group 1 were normal with respect to the blood calcium and inorganic phosphorus and it follows that the chemical composition of the blood of Nigerian Zebu cattle, in so far as these elements are concerned, differs significantly from that of similar animals in East Africa.

Since the animals in Group 2 showed a normal or slightly raised blood picture, it is unlikely that the osteophagia seen in these animals was due to a deficiency of calcium or phosphorus. This result is of some interest as osteophagia is such a constant feature of aphosphorosis as to be considered almost diagnostic of this condition. It may be added that no other evident signs of a phosphorus deficiency have yet been observed either in these or in other cattle; no bone lesions or otherwise unexplainable lameness have been noted and, to date, all bones examined post-mortem have appeared macroscopically normal. I am indebted to Mr. R. S. Marshall, who has had considerable experience of this country, for the information that he has never yet encountered an osteodystrophic condition in Nigerian cattle. In view of the fact that osteophagia was observed during February and March 1949, at the end of an exceptionally long dry season, it seems reasonable to conclude that it is a manifestation of a general starvation rather than of a specific deficiency. In certain of the Provinces where grazing is particularly poor an allotriophagia is not uncommon at such times.

The lowered blood calcium level in animals with trypanosomiasis is of interest, as many of the earths eaten so avidly by these animals contain relatively large amounts of lime (Table III, from Godden, 1929). It is tempting to suppose that the so-called "mudu" pica associated with trypanosomiasis may be connected with a physiological craving for calcium. However, unlike the "normal" pica, "mudu" cannot be successfully counteracted by feeding native salts

TABLE III

THE CALCIUM, MAGNESIUM AND PHOSPHORUS CONTENT OF NIGERIAN EDIBLE EARTHS AND NATIVE SALTS (from Godden, 1929)

Calcium (as CaO)	...	...	...	0.016-2.548, av. 0.658 per cent.
Magnesium (as MgO)	...	...	...	0.032-0.506, av. 0.233 "
Phosphorus (as P ₂ O ₅)	...	...	...	Trace-0.153, av. 0.041 "

and, if it is due to a demand for calcium, it is difficult to understand why this demand cannot be satisfied by the salts, since they also contain a large amount of calcium. From Table III it will be seen that it is unlikely that either form of pica has its origin in a phosphorus deficiency as the phosphorus content of both earths and salts was consistently low.

Fiennes, Jones and Laws (1946) have also observed a tendency for the blood calcium to fall in *T. congolense* infection. French (1938) found that the blood calcium was unaltered in several cases, but showed a tendency to decrease in others. He recalls that a hypocalcaemia has been reported in several acute febrile infectious diseases. Fiennes *et al.* recorded a gradual decrease in inorganic phosphorus, but French is in agreement with the present author in finding no change in this blood constituent.

COPPER

During the rainy season of 1948, Mr. K. Unsworth, while working on helminthiasis in young cattle, observed that they developed a severe anaemia which showed a positive correlation with the rainfall and with the faecal egg-count, but he considered that the worm burden was not sufficiently heavy to account for the severity of the anaemia. A much less severe anaemia has been recorded this year in the same group of animals and in other laboratory cattle, although the faecal egg-counts have fallen to very low values. Cows at the Livestock Improvement Centre receiving concentrates show either no anaemia or, at most, only a slight anaemia, even at the height of lactation. The anaemia is considered to be of the macrocytic hypochromic type, although the degree of hypochromia varies between individual animals.

There seemed to be a striking similarity between this condition and the "falling disease" or "sudden death" recorded by Bennetts and Hall (1939) and Bennetts, Beck, Harley and Evans (1941) as enzootic in South-Western Australia. According to these workers "the clinical symptoms in adult cattle are especially evident during the flush growth of pastures from August to October, when despite this, there is some loss in condition, a rough, staring coat and evidence of anaemia." The periods of heaviest rainfall in those particular areas of South-Western Australia concerned and in Vom are nearly coincidental and the anaemia (and the general condition of the cattle) was at its worst from August to November, 1948. "Falling disease" is associated constantly with "a very low copper content of pastures, a low copper status (blood and liver) of animals in affected herds, and the response to copper alone or in association with other supplements."

Pasture Copper

The copper content of grass collected over the Vom area during June, July and August 1949 was found to vary between 1.2 and 8.3

p.p.m. D.M., with an average of 7.7 p.p.m. Bennetts *et al.* (1941), found the copper content of ordinary mixed pastures where stock remains healthy, to vary from 5 to 15 p.p.m. D.M. The Vom grasses are therefore relatively poor in copper. A further point to be borne in mind is that widespread deposits of tin and more localised occurrences of lead and zinc are to be found on the Plateau, and these elements are suspected to interfere with absorption of copper from the alimentary tract.

Blood Copper

During July 1949, when the anaemia was well established, the serum copper of 25 laboratory animals used in this investigation and of 20 non-anaemic Livestock Improvement Centre cows averaged 0.076 and 0.077 mg. per cent. respectively. The normal range in Australian cattle is given as 0.07 to 0.17 mg. Cu per cent., the blood copper levels of anaemic cows being of the order of 0.01 to 0.02 mg. per cent. (Bennetts *et al.*, 1941).

Response to Copper Supplements

The daily administration of 100 mg. of copper (as the sulphate) produced no response, even when continued over a period of six weeks. It is concluded, therefore, that a deficiency of copper does not contribute to the wet season anaemia.

IRON

Hypochromia is usually associated with a shortage of iron. The soil in this area contains large amounts of iron in the form of haematite and the iron content of the pasture is correspondingly high, varying from 0.05 to 0.13 per cent. D.M. (mean 0.09). This concentration is much higher than is usually found in plants.

In order to obtain as complete a picture as possible, iron was administered to a few animals at the rate of 1 g. ferrous sulphate daily. As might be expected no response was obtained.

Attention has recently been drawn to the influence of the diet on iron absorption with particular reference to the role played by the phosphorus of the diet (Kinney, Hegsted and Finch, 1949, Hegsted, Finch and Kinney, 1949). Diets containing large amounts of iron with a low level of phosphorus led to excessive deposition of iron in the livers of rats. Histological examination of livers from cattle at this laboratory has not revealed any haemosiderosis other than in occasional specimens from animals suffering from chronic trypanosomiasis. It is therefore concluded that the abnormally high concentration of iron found in the Vom pasture has little, if any, pathological significance.

COBALT

A deficiency of cobalt may be accompanied by pica and anaemia; both symptoms have been observed. Further, it was reported from

the Veterinary Station at Kaduna, Northern Nigeria, that many cattle in that district presented an appearance greatly resembling the published photographs of cobalt deficient stock. The geology of the Plateau would seem to favour the occurrence of a cobalt deficiency, since the underlying rock, as in many other parts of Nigeria, is the Pre-Cambrian igneous-metamorphic complex, consisting of granites, gneisses and schists. It is well known that such acid igneous rocks have, in general, a low cobalt (and copper) content and that soils derived from these rocks will be poor in cobalt.

Soil Cobalt

The cobalt content of the soil in the Vom area averages 0.75 p.p.m. soluble in 2.5 per cent. acetic acid. The soil is acid with an average reaction of *pH* 5.9 as measured by the glass electrode. This amount would appear adequate, for Mitchell (1948) considers that a cobalt deficiency is unlikely to occur on an acid mineral soil containing more than 0.25 p.p.m. Co soluble in 2.5 per cent. acetic acid. The possibility still remained that this cobalt might not be available to stock, for little is known of the factors affecting its absorption.

Response to Cobalt Supplements

Cattle drenched daily for six weeks with a solution containing 100 mg. of copper sulphate and 1 mg. of cobalt sulphate showed no improvement in condition.

MANGANESE AND MOLYBDENUM

Vom pastures do not contain abnormal amounts of manganese (20 to 59 p.p.m. D.M.), while molybdenum occurs only in traces too small to be estimated by the techniques available. No trouble is therefore likely to ensue either from a deficiency or excess of manganese or an excess of molybdenum.

DISCUSSION

Despite the presence of symptoms suggestive of mineral deficiency, cattle in the area under consideration appear to receive adequate amounts of calcium, magnesium, phosphorus, copper, cobalt, iron and manganese from the foodstuffs commonly available even under the worst conditions. Osteophagia and geophagia may be forms of a more general allotrophagia associated with an absolute shortage of food rather than with a specific deficiency inherent in the food. The anaemia occurring during the rainy season is still receiving attention and no definite conclusion has yet been reached as to its origin. A more complete account will be published later.

SUMMARY

Evidence is put forward to suggest that deficiencies of calcium, magnesium, phosphorus, copper and cobalt might occur in cattle in

the vicinity of Vom, Plateau Province. Northern Nigeria.

The status of mineral nutrition of stock with regard to these elements and to iron, manganese and molybdenum has been investigated.

No deficiency of the elements under consideration has been detected and no trouble is likely to be encountered from an excess of manganese or molybdenum.

ACKNOWLEDGMENTS

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OBSERVATIONS ON THE VALUE OF SMALL DAILY DOSES OF PHENOTHAZINE FOR THE CONTROL OF TRICHOSTRONGYLOSIS IN SHEEP

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INTRODUCTION

NUMEROUS reports in the literature indicate that small daily doses of phenothiazine, mixed with salt, administered as a lick are effective in the control of trichostrongylosis of sheep. The results of most of the trials reported are, however, assessed on the basis of clinical improvement and in only one or two instances are suggestions made as to the mechanism by which the small doses of phenothiazine exert their useful effect. The laboratory experiments described in this paper were undertaken in the hope of throwing some light on this point, since a fuller understanding of the mode of action of small doses of phenothiazine would probably lead to improvements through rational application of the system of treatment.

Preliminary experiments were carried out on six pairs of twin lambs and subsequently the work was repeated on larger groups. The experimental lambs were reared under special conditions which protected them from infestation with all nematodes excepting *Strongyloides papillosus*. It was also found impracticable to keep them free from coccidial infection, but neither of these infestations appeared to exert any pathogenic effect, and as they were present in all the animals it is thought that they could not have affected the results. Throughout the experiment all the animals were fed on a diet of hay.

PRELIMINARY EXPERIMENTS

The initial trials were designed to demonstrate whether small doses of phenothiazine have any effect on the percentage of larvae which, when picked up by a sheep, develop into adult worms; and whether there is any effect on the adult worms which may be present at the time of treatment.

First Preliminary Experiment

Two pairs of twin lambs nine weeks old were kept on the hay diet for a fortnight without treatment, and then one of each pair was dosed daily for 21 days with one gramme of dispersable phenothiazine suspended in about 10 c.c. of tap water. Immediately afterwards, on every day except the first, all four lambs received 10 c.c. of water containing 2,000 infective larvae of *Trichostrongylus axei*; this routine was continued until each lamb had received 40,000 infective larvae. Faecal egg counts were carried out at weekly intervals during the first four weeks and then daily until

the end of the experiment. The animals were weighed each week and were fasted some 17 hours prior to weighing to prevent aberrant weight changes due to ingestion of large quantities of food and water. The animals were slaughtered 26 days after the last dose of larvae had been administered, and the numbers of *Trichostrongylus axei* present in the abomasum of each lamb were estimated by a dilution technique. The results of the experiments, given in Table 1, show that the egg counts of the two animals receiving one gramme doses of phenothiazine daily were about half those of the untreated animals and their worm burdens showed a corresponding variation. No significant difference in the rate of weight gain was observed as between treated and control lambs.

TABLE I

SHOWING THE CHANGES IN EGG COUNT AND WEIGHT OF THE FOUR LAMBS USED IN THE FIRST PRELIMINARY EXPERIMENT, AND ALSO THE WORM BURDENS OF THE LAMBS AT THE END OF THE EXPERIMENT

Lamb 1090 receiving doses of larvae only		Lamb 1091 receiving doses of phenothiazine and larvae		Lamb 1300 receiving doses of larvae only		Lamb 1299 receiving doses of phenothiazine and larvae			
Date				Date					
	Eggs per g. of faeces	Weight in pounds	Eggs per g. of faeces	Weight in pounds	Eggs per g. of faeces	Weight in pounds	Eggs per g. of faeces	Weight in pounds	
3.7.45	—	42½	—	45	4.6.46	0	34	0	32½
10.7.45	—	45	—	47	11.6.46	0	32	0	33
17.7.45	—	42½	—	45	18.6.46	0	32	0	32
24.7.45	10	47½	0	47½	24.6.46	0	—	0	—
25.7.45	23	—	6	—	25.6.46	0	31	0	32
26.7.45	100	—	100	—	26.6.46	0	—	0	—
27.7.45	500	—	100	—	27.6.46	6	—	0	—
28.7.45	400	—	400	—	28.6.46	100	—	2	—
30.7.45	400	—	400	—	29.6.46	300	—	100	—
31.7.45	500	45	400	46	30.6.46	300	—	200	—
1.8.45	400	—	500	—	1.7.46	600	—	200	—
2.8.45	400	—	400	—	2.7.46	400	31	100	31
3.8.45	1,000	—	300	—	3.7.46	500	—	200	—
4.8.45	700	—	400	—	4.7.46	600	—	300	—
7.8.45	900	46	400	46	5.7.46	700	—	200	—
8.8.45	800	—	500	—	6.7.46	1,200	—	200	—
9.8.45	800	—	500	—	7.7.46	1,200	—	400	—
10.8.45	900	—	600	—	8.7.46	1,100	—	200	—
11.8.45	1,000	—	600	—	9.7.46	1,000	27½	300	27½
13.8.45	800	—	400	—	10.7.46	1,200	—	300	—
14.8.45	900	46	500	47	11.7.46	600	—	200	—
15.8.45	1,000	—	400	—	12.7.46	800	—	300	—
16.8.45	800	—	400	—	13.7.46	700	—	200	—
17.8.45	1,000	—	500	—	14.7.46	800	—	200	—
					15.7.46	700	—	200	—
					16.7.46	700	24	200	24
					17.7.46	900	—	200	—
					18.7.46	1,000	—	400	—
Worm burden									
	10,300		5,500			43,600		27,800	

Second Preliminary Experiment

Since different results might be expected when the doses of phenothiazine are given a few hours before the larvae instead of immediately before, in this second experiment an interval of eight hours was allowed to elapse between the administration of the drug and the larvae. Two pairs of twin lambs were again used, one pair being 12 weeks old and the other pair 16 weeks old at the beginning of the experiment. After a fortnight on the hay diet one lamb in each group was given one gramme of dispersable phenothiazine daily

TABLE II

SHOWING THE CHANGES IN EGG COUNT AND WEIGHT OF THE FOUR LAMBS USED IN THE SECOND PRELIMINARY EXPERIMENT, AND ALSO THE WORM BURDENS OF THE LAMBS AT THE END OF THE EXPERIMENT

Date	Lamb 1572 receiving doses of larvae only		Lamb 1571 receiving doses of phenothiazine and larvae		Date	Lamb 1691 receiving doses of larvae only		Lamb 1690 receiving doses of phenothiazine and larvae	
	Eggs per g. of faeces	Weight in pounds	Eggs per g. of faeces	Weight in pounds		Eggs per g. of faeces	Weight in pounds	Eggs per g. of faeces	Weight in pounds
23.7.46	0	39	0	42½	10.3.47	0	—	0	—
30.7.46	0	36	0	41	13.3.47	—	62½	—	65
6.8.46	0	37	0	43	17.3.47	0	—	0	—
13.8.46	0	37½	0	41½	20.3.47	—	58	—	65
20.8.46	0	40	0	42	24.3.47	0	—	0	—
25.8.46	0	—	0	—	27.3.47	—	60	—	62½
26.8.46	0	—	0	—	31.3.47	0	—	0	—
27.8.46	0	37½	0	37½	3.4.47	—	60	—	65
28.8.46	21	—	9	—	7.4.47	24	—	78	—
29.8.46	64	—	14	—	9.4.47	110	—	155	—
30.8.46	94	—	59	—	10.4.47	—	60	—	62½
31.8.46	300	—	300	—	11.4.47	700	—	200	—
1.9.46	500	—	200	—	14.4.47	900	—	500	—
2.9.46	700	—	600	—	16.4.47	900	—	1,300	—
3.9.46	800	40	500	40	17.4.47	—	60	—	62½
4.9.46	600	—	200	—	18.4.47	700	—	600	—
5.9.46	700	—	300	—	21.4.47	800	—	1,200	—
6.9.46	700	—	400	—	23.4.47	1,500	—	1,900	—
7.9.46	500	—	600	—	24.4.47	—	57½	—	57½
8.9.46	600	—	500	—					
9.9.46	400	—	700	—					
10.9.46	600	40	700	40					
11.9.46	500	—	700	—					
12.9.46	1,600	—	1,200	—					
13.9.46	1,300	—	1,000	—					
14.9.46	1,100	—	600	—					
15.9.46	600	—	500	—					
16.9.46	800	—	500	—					
17.9.46	1,200	42	900	42½					
18.9.46	1,000	—	900	—					
19.9.46	900	—	700	—					
20.9.46	1,800	—	2,300	—					
Worm burden	24,800		18,900			22,950		17,250	

at 8.45 a.m. for 20 days and at 4.45 p.m. each of the four lambs received 2,000 infective larvae of *Trichostrongylus axei*; a total of 40,000 larvae being administered to each lamb. For the first six weeks of the experiment weekly faecal egg counts were carried out and counts were afterwards performed daily on the younger pair of lambs and three times weekly on the other pair. The animals were weighed weekly. Twenty-six days after the last doses of phenothiazine and of larvae had been given, all the lambs were killed and the number of *Trichostrongylus axei* present in the abomasum of each was estimated.

The results of this experiment are given in Table II, a perusal of which shows that the egg counts of the treated lambs were slightly lower than those of the controls and that worm burdens were also slightly lower. There was no difference in the rate of weight gain in the treated and control lambs.

Third Preliminary Experiment

Again two pairs of twin lambs were used, one pair 12 weeks old and the other pair 16 weeks old at the beginning of the observation. After receiving the hay diet for a fortnight all four lambs were given daily doses of 4,000 infective larvae of *Trichostrongylus axei* until 80,000 larvae had been administered to each lamb. Four weeks after the last dose of larvae had been given, by which time all the larvae would have developed into mature worms, one of each pair of lambs was given daily one gramme doses of dispersable phenothiazine suspended in 10 c.c. of tap water. These daily doses of phenothiazine were continued for 20 consecutive days. Egg counts were carried out daily on faeces collected from the younger pair of lambs beginning at the time when dosing with larvae was completed. Faecal samples were examined weekly for the first five weeks of the experiment and then three times weekly until observations ceased. The lambs were weighed each week. Five days after the last dose of phenothiazine had been given all the lambs were slaughtered and the number of *Trichostrongylus axei* present in the abomasum of each estimated.

The results of this experiment are given in Table III, which shows that there was no difference between the worm burdens of the treated and control lambs and no significant difference in the rate of weight gain of the four lambs.

The preliminary experiments described above show that daily one gramme doses of phenothiazine do not prevent infective trichostrongylid larvae from developing to maturity in treated sheep unless the larvae and phenothiazine are swallowed within a short time of each other when the phenothiazine is partially effective. It was also shown that daily one gramme doses had no effect in reducing infestations of mature worms in lambs. It may be concluded, therefore, that small doses of phenothiazine must exert their useful effect in some way other than by direct action on ingested larvae

TABLE III

SHOWING THE CHANGES IN EGG COUNT AND WEIGHT OF THE FOUR LAMBS USED IN THE THIRD PRELIMINARY EXPERIMENT, AND ALSO THE WORM BURDENS OF THE LAMBS AT THE END OF THE EXPERIMENT

Date	Lamb 1568 receiving doses of larvae only		Lamb 1569 receiving doses of phenothiazine and larvae		Date	Lamb 1693 receiving doses of larvae only		Lamb 1692 receiving doses of phenothiazine and larvae	
	Eggs per g. of faeces	Weight in pounds	Eggs per g. of faeces	Weight in pounds		Eggs per g. of faeces	Weight in pounds	Eggs per g. of faeces	Weight in pounds
23.7.46	—	41	—	42½	10.3.47	0	—	0	—
30.7.46	—	44	—	40	13.3.47	—	67½	—	60
6.8.46	—	45	—	42½	17.3.47	0	—	0	—
13.8.46	—	47	—	42½	20.3.47	—	72½	—	60
20.8.46	—	45	—	42½	24.3.47	0	—	0	—
25.8.46	—	—	—	—	27.3.47	—	67½	—	57½
26.8.46	33	—	16	—	31.3.47	1	—	1	—
27.8.46	103	43	53	42½	3.4.47	—	70	—	57
28.8.46	100	—	85	—	7.4.47	44	—	59	—
29.8.46	196	—	99	—	9.4.47	245	—	600	—
30.8.46	200	—	200	—	10.4.47	—	68	—	55
31.8.46	500	—	400	—	11.4.47	500	—	700	—
1.9.46	400	—	600	—	14.4.47	800	—	900	—
2.9.46	900	—	400	—	16.4.47	1,600	—	2,500	—
3.9.46	800	45	900	42½	17.4.47	—	70	—	57½
4.9.46	900	—	500	—	18.4.47	1,300	—	2,800	—
5.9.46	700	—	900	—	21.4.47	1,900	—	2,000	—
6.9.46	700	—	1,100	—	23.4.47	3,500	—	5,800	—
7.9.46	1,100	—	1,000	—	24.4.47	—	66	—	57½
8.9.46	1,100	—	500	—	25.4.47	1,700	—	5,600	—
9.9.46	1,000	—	1,100	—	28.4.47	1,800	—	2,700	—
10.9.46	1,100	45	900	44	30.4.47	1,500	—	3,900	—
11.9.46	900	—	900	—	1.5.47	—	72½	—	55
12.9.46	1,100	—	1,100	—	2.5.47	1,400	—	3,200	—
13.9.46	1,800	—	1,700	—	5.5.47	1,900	—	2,700	—
14.9.46	1,200	—	1,100	—	7.5.47	2,000	—	2,000	—
15.9.46	1,000	—	900	—	8.5.47	—	70	—	52½
16.9.46	700	—	900	—	9.5.47	1,700	—	3,100	—
17.9.46	800	47½	300	44	12.5.47	900	—	2,000	—
18.9.46	700	—	600	—	14.5.47	900	—	2,300	—
19.9.46	1,000	—	600	—	15.5.47	—	67½	—	50
20.9.46	1,400	—	800	—	16.5.47	1,300	—	2,500	—
21.9.46	900	—	400	—	19.5.47	900	—	3,200	—
22.9.46	800	—	400	—	21.5.47	1,400	—	3,500	—
23.9.46	1,000	—	200	—	22.5.47	—	62	—	47½
24.9.46	1,000	47½	500	45					
25.9.46	1,000	—	600	—					
26.9.46	1,200	—	500	—					
27.9.46	1,400	—	600	—					
28.9.46	700	—	400	—					
29.9.46	700	—	300	—					
30.9.46	900	—	200	—					
1.10.46	1,500	47½	600	46					
2.10.46	800	—	200	—					
3.10.46	700	—	100	—					
4.10.46	700	—	200	—					
5.10.46	900	—	200	—					
6.10.46	600	—	200	—					
7.10.46	1,100	—	500	—					
8.10.46	1,600	46	500	42½					

TABLE III—continued

Date	Lamb 1568 receiving doses of larvae only		Lamb 1569 receiving doses of phenothiazine and larvae		Date	Lamb 1693 receiving doses of larvae only		Lamb 1692 receiving doses of phenothiazine and larvae	
	Eggs per g. of faeces	Weight in pounds	Eggs per g. of faeces	Weight in pounds		Eggs per g. of faeces	Weight in pounds	Eggs per g. of faeces	Weight in pounds
9.10.46	1,400	—	400	—					
10.10.46	1,200	—	400	—					
11.10.46	1,300	—	400	—					
12.10.46	1,500	—	400	—					
13.10.46	1,200	—	300	—					
14.10.46	1,000	—	200	—					
15.10.46	1,500	47½	300	45					
16.10.46	1,600	—	600	—					
17.10.46	2,100	—	800	—					
Worm burden	31,200		32,000			27,550		27,100	

or on mature worms in lambs, namely, through preventing the development of infective larvae in the faeces of treated animals. This effect which has been demonstrated by several workers (Taylor and Sanderson, 1940; Shorb and Habermann, 1940; Habermann and Shorb, 1942; Gibson, 1945 and 1949) was, therefore, considered in the confirmatory experiments which were planned to verify the results already obtained.

CONFIRMATORY EXPERIMENT

This experiment was carried out in two parts, the second being a repetition of the first.

Six pairs of twin lambs were used on each occasion. At the beginning of the period of observation those in the first part of the experiment were eight months old and those in the second part four months old. After a fortnight on the diet of hay only, one of each pair of lambs was given one gramme of dispersable phenothiazine at 9.0 a.m. and all 12 lambs were given 4,000 infective larvae of *Trichostrongylus axei* at 4.45 p.m. every day from Monday to Friday of each week, neither phenothiazine nor larvae being given on Saturday or Sunday. Weights and faecal egg counts were recorded at weekly intervals. When an egg count was found to exceed ten per gramme, a faecal culture was made and after incubation at 27° C. for seven days, the infective larvae were separated from the culture by means of the Baermann apparatus. The larvae so obtained were counted, either by examination of the whole culture when only a few were present or by the dilution method when they were numerous. The yield of larvae from a given culture was compared with the theoretical expectation calculated from the egg count and

the weight of faeces in the culture. The experiment was carried on in this way until 16 weeks of dosing had been completed, after which all surviving lambs were slaughtered and the number of worms present in the abomasum and small intestine of each was estimated in the usual way.

RESULTS

The results of the first part of the confirmatory experiment are set out in Tables IV and V. Table IV shows the figures for the sheep receiving daily doses of phenothiazine and Table V those for their twins in the untreated control group. From these tables it will be observed that all the sheep survived to the end of the experiment and that there was little difference in weight gain, worm burden or egg counts between the two groups. There was, however, a striking difference between the two groups in the percentage of larvae recovered from a 20 g. faecal culture compared with the theoretical expectation of larvae calculated from the egg count. In the group receiving phenothiazine the percentage of larvae was zero except on four occasions when it was less than 1 per cent. In the control group it ranged from 10 to 100 per cent. but was usually in the region of 20 to 40 per cent.; this latter range represented about the average recovery of larvae from routine faecal culture. There was, therefore, no doubt of the potent effect of a daily dose of one gramme of phenothiazine in inhibiting larval development in the faeces.

Tables VI and VII giving the results of the second part of the confirmatory experiment for the treated and control groups respectively show that the group receiving phenothiazine treatment was as a whole rather better than the control group. There were two deaths in the treated group and four in the control group. The loss of weight over the whole period was less in the treated group and the total worm burden of the treated group was about half that of the control group. Although the treated group were better than the controls they did suffer severe loss of weight and carried a heavy worm burden and the treatment was, therefore, not effective in preventing the development of parasitic gastritis in the group. It will again be noticed that the small daily doses of phenothiazine prevented the development of infective larvae from eggs passed in the faeces of the treated sheep.

DISCUSSION

It will be observed that in the first preliminary experiment where the daily doses of phenothiazine and larvae were given to the lambs within a few moments of each other and also in the second part of the confirmatory experiment, the egg counts of lambs in the treated groups were lower than those of the lambs in the control groups. In all other experiments there was no difference in counts between the two groups. This result is in marked contrast to that

TABLE IV

SHOWING THE RESULTS OBTAINED IN THE FIRST PART OF THE CONFIRMATORY EXPERIMENT FOR THE SHEEP RECEIVING DAILY DOSES OF BOTH PHENOTHIAZINE AND LARVAE

1694					1696			
Date	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture	
			Actual number	Expected number			Actual number	Expected number
31.7.47	105	0	—	—	96	0	—	—
7.8.47	107	0	—	—	95	0	—	—
14.8.47	107½	0	—	—	96	0	—	—
21.8.47	102½	0	—	—	92½	0	—	—
28.8.47	98	0	—	—	92½	0	—	—
4.9.47	98	0	—	—	92½	0	—	—
11.9.47	100	48	0	960	92½	11	0	220
18.9.47	104	100	0	2,000	90	47	0	940
25.9.47	100	300	0	6,000	92½	100	0	2,000
2.10.47	100	100	0	2,000	95	100	0	2,000
9.10.47	102½	62	0	1,240	95	28	0	560
16.10.47	100	100	0	2,000	92	200	0	4,000
23.10.47	100	25	0	500	92½	7	0	140
30.10.47	100	5	0	100	87	9	0	180
6.11.47	102½	2	6	40	87½	5	0	100
13.11.47	102½	2	—	—	91	2	—	—
20.11.47	105	7	—	—	90	3	—	—
29.11.47	102½	1	—	—	95	7	—	—
Worm burden		19,100			42,700			
1721					1724			
Date	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture	
			Actual number	Expected number			Actual number	Expected number
31.7.47	112½	0	—	—	90	0	—	—
7.8.47	112½	0	—	—	90	0	—	—
14.8.47	112½	0	—	—	87½	0	—	—
21.8.47	110	0	—	—	85	0	—	—
28.8.47	104	0	—	—	82½	0	—	—
4.9.47	105	1	—	—	85	1	—	—
11.9.47	105	14	0	280	85	45	0	900
18.9.47	106	100	0	2,000	82½	100	0	2,000
25.9.47	102½	300	0	6,000	81	100	0	2,000
2.10.47	102½	100	0	2,000	85	200	0	4,000
9.10.47	105	200	0	4,000	86	200	0	4,000
16.10.47	101	100	0	2,000	85	100	0	2,000
23.10.47	102½	4	0	80	82	2	0	40
30.10.47	100	8	0	160	82½	4	0	80
6.11.47	102½	6	0	120	81	6	0	120
13.11.47	105	4	—	—	82	11	—	—
20.11.47	102½	2	—	—	85	13	—	—
29.11.47	102½	1	—	—	82½	1	—	—
Worm burden		18,400			15,800			

TABLE IV—continued

Date	1732				1780			
	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture	
			Actual number	Expected number			Actual number	Expected number
31.7.47	100	0	—	—	95	0	—	—
7.8.47	97½	0	—	—	92½	0	—	—
14.8.47	94	0	—	—	98	0	—	—
21.8.47	92½	0	—	—	92½	0	—	—
28.8.47	92½	0	—	—	90	0	—	—
4.9.47	92½	1	—	—	90	3	—	—
11.9.47	90	85	0	1,700	87½	71	2	1,420
18.9.47	90	200	0	4,000	92½	400	1	8,000
25.9.47	90	100	0	2,000	86	200	0	4,000
2.10.47	92	100	0	2,000	92	400	0	8,000
9.10.47	92½	300	0	6,000	90	100	0	2,000
16.10.47	90	100	0	2,000	89	100	0	2,000
23.10.47	87½	8	0	160	88	55	12	1,100
30.10.47	85	7	0	140	85	25	0	500
6.11.47	87½	8	0	160	87½	50	0	1,000
13.11.47	87½	5	—	—	85	66	—	—
20.11.47	90	4	—	—	87½	32	—	—
29.11.47	90	10	—	—	85	35	—	—
Worm burden		18,250			56,600			

NOTE.—First dose of phenothiazine and larvae given 11.8.47.
 Last " " " " 28.11.47.

TABLE V

SHOWING THE RESULTS OBTAINED IN THE FIRST PART OF THE CONFIRMATORY
 EXPERIMENT FOR THE SHEEP RECEIVING DAILY DOSES OF LARVAE ONLY

Date	1695				1697			
	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture	
			Actual number	Expected number			Actual number	Expected number
31.7.47	92½	0	—	—	99	0	—	—
7.8.47	90	0	—	—	95	0	—	—
14.8.47	90	0	—	—	92½	0	—	—
21.8.47	87½	2	—	—	95	0	—	—
28.8.47	85	5	—	—	92	0	—	—
4.9.47	85	8	—	—	90	0	—	—
11.9.47	85	68	700	1,360	87½	51	800	1,020
18.9.47	82½	700	3,050	14,000	90	500	1,900	10,000
25.9.47	84	100	800	2,000	90	200	3,200	4,000
2.10.47	86	100	850	2,000	87½	200	1,900	4,000
9.10.47	87½	200	1,100	4,000	92½	300	1,650	6,000
16.10.47	86	100	200	2,000	92½	200	2,200	4,000
23.10.47	80	8	71	160	92½	200	1,000	4,000
30.10.47	80	2	6	40	87½	48	250	960
6.11.47	84	1	3	20	90	30	300	600
13.11.47	82½	2	—	—	87½	17	—	—
20.11.47	86	0	—	—	90	60	—	—
29.11.47	87	0	—	—	92½	12	—	—
Worm burden		8,550			23,400			

TABLE V—*continued*

Date	1722				1725			
	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal cultures	
			Actual number	Expected number			Actual number	Expected number
31.7.47	85	0	—	—	115	0	—	—
7.8.47	82½	0	—	—	113	0	—	—
14.8.47	82½	0	—	—	114	0	—	—
21.8.47	80	0	—	—	110	0	—	—
28.8.47	77½	0	—	—	106	6	—	—
4.9.47	80	5	—	—	110	16	—	—
11.9.47	77½	262	2,550	5,240	107½	234	2,800	4,680
18.9.47	77½	300	700	6,000	107½	400	2,800	8,000
25.9.47	76	300	2,200	6,000	107½	200	6,500	4,000
2.10.47	72½	700	3,000	14,000	105	600	3,000	12,000
9.10.47	76	300	4,850	6,000	107½	700	6,800	14,000
16.10.47	75	100	2,300	2,000	110	500	4,150	10,000
23.10.47	72½	12	650	240	107½	400	2,500	8,000
30.10.47	68	73	300	1,460	105	122	3,000	2,440
6.11.47	72½	38	350	760	102½	113	1,700	2,260
13.11.47	67½	127	—	—	102½	69	—	—
20.11.47	75	24	—	—	105	85	—	—
29.11.47	76	50	—	—	105	287	—	—
Worm burden			25,250				41,800	

Date	1733				1781			
	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture	
			Actual number	Expected number			Actual number	Expected number
31.7.47	80	0	—	—	100	0	—	—
7.8.47	80	0	—	—	97	0	—	—
14.8.47	80	0	—	—	97½	0	—	—
21.8.47	77½	5	—	—	95	0	—	—
28.8.47	75	24	—	—	92½	2	—	—
4.9.47	80	37	—	—	95	19	—	—
11.9.47	73	546	2,800	10,920	95	154	1,250	3,080
18.9.47	77½	800	6,250	16,000	92½	400	2,000	8,000
25.9.47	76	300	3,650	6,000	90	300	3,800	6,000
2.10.47	78	100	1,650	2,000	92	200	3,150	4,000
9.10.47	82½	400	2,100	8,000	95	100	5,200	2,000
16.10.47	82	100	900	2,000	95	200	2,000	4,000
23.10.47	78	4	143	80	89	200	2,450	4,000
30.10.47	77½	2	38	40	85	247	3,000	4,940
6.11.47	79	2	0	40	90	47	900	940
13.11.47	82½	0	—	—	87½	42	—	—
20.11.47	85	0	—	—	90	49	—	—
29.11.47	80	5	—	—	90	37	—	—
Worm burden			6,300				30,200	

NOTE.—First dose larvae given 11.8.47. Last dose larvae given 28.11.47.

TABLE VI
SHOWING THE RESULTS OBTAINED IN THE SECOND PART OF THE CONFIRMATORY
EXPERIMENT FOR THE SHEEP RECEIVING DAILY DOSES OF BOTH PHENOTHIAZINE
AND LARVAE

Date	1859				1867			
	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture	
			Actual number	Expected number			Actual number	Expected number
24.3.48	—	0	—	—	—	0	—	—
31.3.48	65	0	—	—	52½	0	—	—
7.4.48	65	0	—	—	50	0	—	—
14.4.48	65	0	—	—	51	0	—	—
21.4.48	65	0	—	—	50	0	—	—
28.4.48	62½	2	—	—	50	7	—	—
5.5.48	62½	200	0	4,000	47½	600	0	12,000
12.5.48	60	300	0	6,000	46	600	0	12,000
19.5.48	52½	200	0	4,000	45	500	0	10,000
26.5.48	55	500	0	10,000	45	500	0	10,000
2.6.48	52½	700	0	14,000	47½	1,200	0	24,000
9.6.48	46	800	0	16,000	46	1,000	0	20,000
16.6.48	48	800	0	16,000	47½	1,000	0	20,000
23.6.48	40	1,400	0	28,000	44	800	0	16,000
30.6.48	36½	1,600	0	32,000	39	1,300	0	26,000
7.7.48	39	1,100	0	22,000	41	600	0	12,000
14.7.48	40½	1,400	0	28,000	41½	1,600	0	32,000
Worm burden			30,200				58,000	

Date	1869				1872			
	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture	
			Actual number	Expected number			Actual number	Expected number
24.3.48	—	0	—	—	—	0	—	—
31.3.48	72½	0	—	—	75	0	—	—
7.4.48	70	0	—	—	67½	0	—	—
14.4.48	71	0	—	—	72	0	—	—
21.4.48	70	0	—	—	70	0	—	—
28.4.48	71	35	—	—	72½	13	—	—
5.5.48	67½	400	0	8,000	70	400	0	8,000
12.5.48	65	400	0	8,000	67½	500	0	10,000
19.5.48	57½	300	—	—	67½	500	0	10,000
26.5.48	57½	200	0	4,000	68	500	0	10,000
2.6.48	55	400	0	8,000	55	1,200	0	24,000
9.6.48	52½	300	0	6,000	62½	1,100	0	22,000
16.6.48	52	100	—	—	65	1,100	0	22,000
23.6.48	42½	300	0	6,000	60	1,000	0	20,000
30.6.48	39	300	0	6,000	53½	700	0	14,000
7.7.48	37½	100	—	—	58½	700	0	14,000
14.7.48		Killed 9.7.48			58	800	0	16,000
Worm burden			13,700				70,150	

TABLE VI—*continued*

Date	1876				1883			
	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture	
			Actual number	Expected number			Actual number	Expected number
24.3.48	—	0	—	—	—	0	—	—
31.3.48	60	0	—	—	72½	0	—	—
7.4.48	55	0	—	—	67½	0	—	—
14.4.48	57½	0	—	—	68	0	—	—
21.4.48	55	0	—	—	67½	0	—	—
28.4.48	55	112	—	—	68	25	—	—
5.5.48	50	300	0	6,000	67½	400	0	8,000
12.5.48	47½	900	0	18,000	62½	1,400	0	28,000
19.5.48	45	400	—	—	60	600	0	12,000
26.5.48	Died 26.5.48				55	100	0	2,000
2.6.48					45	100	0	2,000
9.6.48					55	1,000	0	20,000
16.6.48					59	1,400	0	28,000
23.6.48					50	1,400	0	28,000
30.6.48					45½	1,400	0	28,000
7.7.48					46	1,500	0	30,000
14.7.48					45½	1,700	0	34,000
Worm burden			29,700				42,200	

NOTE.—First doses of phenothiazine and larvae given 5.4.48.
 Last „ „ „ 13.7.48.

TABLE VII

SHOWING THE RESULTS OBTAINED IN THE SECOND PART OF THE CONFIRMATORY
 EXPERIMENT FOR THE SHEEP RECEIVING DAILY DOSES OF LARVAE ONLY

Date	1860				1868			
	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture	
			Actual number	Expected number			Actual number	Expected number
24.3.48	—	0	—	—	—	0	—	—
31.3.48	85	0	—	—	70	0	—	—
7.4.48	77½	0	—	—	65	0	—	—
14.4.48	78	0	—	—	66	0	—	—
21.4.48	77½	0	—	—	65	0	—	—
28.4.48	75	800	—	—	60	100	—	—
5.5.48	80	1,500	10,250	30,000	57½	1,700	15,600	34,000
12.5.48	75	2,500	13,400	50,000	55	2,400	22,800	48,000
19.5.48	76	2,300	20,550	46,000	51	2,200	18,800	44,000
26.5.48	80	3,400	19,900	68,000	53	3,300	13,350	66,000
2.6.48	70	3,400	32,650	68,000	47½	2,600	18,200	32,000
9.6.48	75	2,700	25,300	54,000	52½	2,000	18,500	40,000
16.6.48	77½	2,800	27,500	56,000	52½	1,200	—	—
23.6.48	67½	4,000	29,500	80,000	35	21,000	—	—
30.6.48	63	4,600	36,700	92,000	Died 27.6.48			
7.7.48	66	3,300	23,000	66,000				
14.7.48	66	2,000	26,100	40,000				
Worm burden			84,400				94,000	

TABLE VII—continued

Date	1870				1871			
	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture	
			Actual number	Expected number			Actual number	Expected number
24.3.48	—	0	—	—	—	0	—	—
31.3.48	81	0	—	—	72½	0	—	—
7.4.48	75	0	—	—	65	0	—	—
14.4.48	76	0	—	—	63	0	—	—
21.4.48	75	0	—	—	65	0	—	—
28.4.48	72½	200	—	—	62	500	—	—
5.5.48	72½	1,000	16,100	20,000	60	700	3,800	14,000
12.5.48	72½	1,300	11,300	26,000	55	500	1,550	10,000
19.5.48	70	2,100	23,700	42,000	50	200	—	—
26.5.48	75	1,700	11,000	34,000	41	—	—	—
2.6.48	67½	1,500	14,900	30,000	Died 28.5.48			
9.6.48	71	1,400	5,900	28,000				
16.6.48	72½	1,400	19,200	28,000				
23.6.48	65	1,400	15,800	28,000				
30.6.48	60½	1,900	18,600	38,000				
7.7.48	63	1,500	8,100	30,000				
14.7.48	60½	1,100	15,100	22,000				
Worm burden	74,750				15,500			
Date	1877				1884			
	Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture		Weight in pounds	Eggs per g. of faeces	Larvae from a 20 g. faecal culture	
			Actual number	Expected number			Actual number	Expected number
24.3.48	—	0	—	—	—	0	—	—
31.3.48	57½	0	—	—	72½	0	—	—
7.4.48	50	0	—	—	62½	0	—	—
14.4.48	48	0	—	—	64	0	—	—
21.4.48	50	0	—	—	62½	0	—	—
28.4.48	47½	300	—	—	62½	300	—	—
5.5.48	43	1,400	—	—	60	1,800	17,600	36,000
12.5.48	39	3,200	26,000	64,000	57½	6,000	45,200	12,000
19.5.48	39	4,200	—	—	60	5,000	50,050	100,000
26.5.48	Died 25.5.48				65	5,100	34,800	102,000
2.6.48					62½	6,500	81,400	130,000
9.6.48					57½	5,700	33,400	114,000
16.6.48					61	4,400	25,200	88,000
23.6.48					52½	4,200	49,000	84,000
30.6.48					46½	5,000	4,600	100,000
7.7.48					Died 6.7.48			
Worm burden	73,400				102,450			

NOTE.—First dose larvae given 5.4.48. Last dose larvae given 13.7.48.

which obtains in horses affected with *Trichonema spp.*, for when they are given daily doses of one gramme of phenothiazine the egg count in the faeces is reduced to zero about a fortnight after the beginning of treatment and remains negative for the rest of the treatment period and for several weeks afterwards (Gibson, 1945 and 1949). It is conceivable that in the lambs the duration of the preliminary experiments was too short for this effect to be shown, but this can hardly be advanced for the confirmatory experiments, each of which extended over 16 weeks. There are many reasons why different species of nematode, living in different organs of different species of host animal, should behave differently when exposed to daily treatments with small doses of phenothiazine, but in view of the meagre data available speculation on the cause of these differences would be unprofitable.

A study of Tables IV, V, VI, and VII reveals some interesting features of helminth immunity. The animals used in the first part of the experiment (Tables IV and V) were mature lambs and it is clear that they were able to resist the effects of a heavy infestation of *Trichostrongylus axei*. The egg counts of these lambs rose after infestation with *Trichostrongylus axei* and then fell again as resistance became established so that at the time of post-mortem examination the egg counts were very low in spite of the fact that the lambs were harbouring from 10,000 to 50,000 worms. These animals must, therefore, have depressed the egg-laying powers of the worms almost to zero. This effect was no doubt due to the development of an age immunity in the lambs since they had not previously been exposed to worm infestation. The group of lambs used in the second part of the experiment (Tables VI and VII) were only four months old at the beginning of the experiment. They showed much less resistance to infection and six of them died. All the animals had high egg counts and at the end their worm burdens ranged between 13,000 and 102,000. It seems clear, therefore, that age resistance had not developed at the time when these lambs were exposed to infestation and as a result the effects on them were severe. The experiment indicates that age resistance develops somewhere between four and eight months of age, a result in keeping with the belief that resistance develops in lambs at about weaning time.

It is clear from the observations recorded in this paper that small daily doses of phenothiazine are of no value in reducing the worm burden of sheep carrying infestation of mature *Trichostrongylus axei* and that they will not prevent the appearance of parasitic gastritis in sheep exposed to heavy, daily, larval intake, but these small doses will prevent the contamination of pastures by the inhibition of larval development from eggs passed in the faeces. The importance of this latter point has been emphasised by a number of workers and has been demonstrated by Harbour, Morgan, Sloan and Rayski (1946). They showed that sheep receiving phenothiazine-salt licks in the field gained weight at a greater rate

than controls, that the egg counts of treated sheep were lower than those of controls and that the number of trichostrongylid larvae per pound of herbage on the field grazed by the treated sheep was lower than the number on the herbage of the field upon which the controls were running. These authors considered that the treatment of sheep with phenothiazine salt mixture might prove useful in keeping pasture infestation at a low level. Pollard, Owen and Evans (1949) have also reported on the power of phenothiazine-salt licks in reducing pasture infestation and they also noted a reduction in egg count and worm burden compared with controls. Some authors, however, have reported anthelmintic effects against *Haemonchus contortus* when using small daily doses of phenothiazine in sheep. For example, Gordon (1942) showed that daily doses of 0.4 to 1.4 g. of phenothiazine would control infestation of *H. contortus*, and Page (1949) showed that a daily intake of 0.5 g. of phenothiazine had a marked anthelmintic effect on lambs carrying heavy infestations of *H. contortus* and also that such doses would protect non-immune lambs against a single massive dose of larvae. There appears, therefore, to be a distinct difference in the way different species of nematode react to small daily doses of phenothiazine.

There are many reports, chiefly from the U.S.A. and generally based on clinical improvement, which testify to the good results which may follow the use of phenothiazine-salt licks in the field, and no doubt these results are to a considerable degree due to the power of small daily doses of phenothiazine to inhibit larval development in the faeces. Because of this feature, phenothiazine-salt licks can be usefully applied to control parasitic gastritis where lambs and ewes are running together, since the lambs are then exposed to a much lower pasture infestation. The technique could also be used to control parasitic gastritis in weaned lambs. Complete success would depend on all the sheep consuming the daily doses of phenothiazine, for a few untreated sheep in the flock might contaminate the pasture sufficiently heavily for the lambs to become infected. These latter, being highly susceptible, would soon build up a heavy infestation and would contaminate the pasture at a heavy rate, unless they also learnt to take the phenothiazine-medicated lick or food at an early age. This is a very real difficulty, for in this country, where salt feeding is not a routine, sheep have to be educated for some time before they will take the lick regularly. It would be well, therefore, when using small daily doses of phenothiazine to control parasitic gastritis, to watch the sheep carefully for a while to see that all are taking medicated food or lick regularly.

SUMMARY

Three preliminary experiments each involving two pairs of twin lambs, and a confirmatory experiment in each of the two parts of which six pairs of twin lambs were used, were carried out to ascertain the effect of daily one grammic doses of phenothiazine on the rate

of acquisition of infestation and on the worm burden of lambs kept at a constant rate of artificial infestation with *Trichostrongylus axei*.

When a daily dose of one gramme of phenothiazine is given to lambs within a few minutes of a dose of infective trichostrongylid larvae the treatment does appear to have some effect in reducing the number of infective larvae which develop to maturity. When there is an interval of several hours between the dose of phenothiazine and the dose of larvae each day, the treatment has no effect on the establishment of the larvae in the host. Daily doses of one gramme of phenothiazine have no effect in reducing the number of mature worms present in sheep. Daily doses of one gramme of phenothiazine do not prevent the development of severe parasitic gastritis in sheep continually exposed to infestation when there is an interval of several hours between the administration of the phenothiazine and the consumption of the larvae. Daily doses of one gramme of phenothiazine prevent the development of infective trichostrongylid larvae from eggs passed in the faeces of treated sheep. The effect of daily doses of one gramme of phenothiazine on the egg count in treated sheep was not constant. In some experiments no effect was observed, but in others the treated sheep had a lower egg count than the control group.

It is suggested that the good results reported from the field from the use of phenothiazine-salt mixture in the control of parasitic gastritis must be referable to a reduction in the pasture larval count due to the property of small daily doses of phenothiazine of preventing the development of infective larvae from eggs passed in the faeces and not to an actual reduction in worm burden. It is probable, therefore, that good effects will be produced in lambs running with ewes which are kept on phenothiazine-salt treatment, but that adult sheep already infected are unlikely to benefit from such treatment. The efficacy of the treatment may be vitiated if a substantial number of the sheep refuse to take the medicament and the flock should be watched carefully to ascertain whether certain sheep habitually refuse the lick.

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LEAD AS A NUTRITIONAL HAZARD TO FARM ANIMALS

I. THE DETERMINATION OF LEAD IN BIOLOGICAL MATERIAL

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INTRODUCTION

THE completed studies from this laboratory on lead as a nutritional hazard to farm animals may be conveniently divided into five papers, the first of which deals with the analytical method employed. The principle used is an old one and the main novelty claimed for this "Weybridge Modification" is the simplicity of the technique and the speed with which it can be performed. Almost the entire operation is carried out in a single test-tube. With blood and soft tissues, which are treated direct, it is possible for a single operator to get through 48 determinations (or 24 in duplicate) in a normal working day, or to report on a single sample for diagnostic purposes within an hour of receipt.

"Dithizone," or diphenylthiocarbazone was shown by Fischer (1925) to form many metallic complexes, most of which have a characteristic colour in chloroform or carbon tetrachloride solution. Numerous variations of the "dithizone" method for the determination of lead have been published, some of which have been reviewed by Jacobs (1944). The earlier routine method used in this laboratory for toxicological purposes was a rapid modification of that of Fischer and Leopoldi (1933, 1934, 1940) taking into account the observations of Winter, Robinson, Lamb and Miller (1935). This was altered to its present form about five years ago to cope with work on the metabolism of lead, and has been found satisfactory in many thousands of varied analyses over the ensuing years.

GENERAL METHOD

Principle

The material is subjected to rapid wet oxidation in a pyrex test-tube. Ammonium citrate is added to prevent precipitation of earthy phosphates and interference by iron and the solution is rendered alkaline with ammonia. Cyanide is added to prevent interference by other metals, chloroform and dithizone are added and the excess reagent removed by alkaline cyanide. The extinction coefficient of the chloroform layer is then read photometrically.

*Reagents**Sulphuric Acid A.R.*

Perchloric Acid A.R. (60 per cent. to 72 per cent.).—A good "lead-free" grade is usually purchasable but each consignment should be carefully tested to ensure the lowest possible "blank."

Nitric Acid A.R.—This reagent often has to be redistilled by the operator.

Ammonium Hydroxide A.R. (0.88 per cent.).—Usually obtainable sufficiently free of lead.

Chloroform A.R. (Phosgene free).

Diphenylthiocarbazone (0.1 per cent.).—This is prepared by dissolving 0.1 g. commercial "Dithizone" in 100 ml. chloroform, transferring to a large separating funnel and extracting with successive 100 ml. portions of a 1 per cent. ammonium hydroxide solution to remove impurities. The ammoniacal extracts are combined in a large flask and about 90 ml. sulphurous acid containing 5 per cent. SO_2 added until the orange colour is replaced by a dark greenish-brown precipitate. 100 ml. of chloroform are then added and the whole shaken vigorously. After transfer to a separating funnel, the chloroform solution of dithizone is run off into a dark bottle and preserved under a layer of sulphurous acid.

Ammonium Citrate (50 per cent.).—The purchasable salt is usually too high in lead and the reagent is best prepared by dissolving 500 g. of citric acid in 800 ml. of distilled water and carefully adding about 450 ml. of ammonium hydroxide (see above) with constant stirring and cooling. The addition of ammonium hydroxide is continued to neutrality to brom-thymol blue and the cool solution is then extracted in a separating funnel with successive proportional quantities of chloroform and dithizone reagent until all lead complex is removed and the last extract is green. It is finally decolourised by extraction with pure chloroform and filtered through Whatman 40 filterpaper.

Potassium Cyanide Solution (10 per cent.).—10 g. of potassium cyanide A.R. are dissolved in 100 ml. distilled water.

Cyanide-Citrate-Ammonia Solution.—80 ml. of the 10 per cent. potassium cyanide solution, 3 ml. of the 50 per cent. ammonium citrate solution and 10 ml. of the 0.88 per cent. ammonium hydroxide are diluted to 1,000 ml. with distilled water.

Cyanide-Ammonia Wash Solution.—10 ml. of 10 per cent. potassium cyanide solution and 10 ml. ammonium hydroxide are diluted to 1,000 ml. with distilled water.

Standard Lead Solution.—To 183.1 mg. lead acetate A.R. are added 3 ml. glacial acetic acid A.R., with sufficient distilled water to form a solution which is finally made up to exactly 1,000 ml.; 1 ml. of this stock solution contains 0.1 mg. Pb. It is diluted as required for making up photometric standards.

Procedure

A sample of the material to be analysed equivalent to not more than 2 g. dry matter is introduced into an 8 in. by 1 in. Pyrex test tube scratched at approximately 10 ml. and 25 ml. levels: 1 ml. sulphuric acid, 3 ml. perchloric acid and three small glass beads are added. Combustion with nitric acid added *guttatim* is carried out according to the method described by Allcroft and Green (1937) for manganese and by Eden and Green (1940) in more detail for copper. The cooled digest is diluted to about 10 ml. with distilled water and boiled for about half a minute to effect solution of deposited solid. 3 ml. of 50 per cent. ammonium citrate solution are added and if at this stage the solution is not clear it is boiled again. It is then diluted

with water in the same tube to approximately the 25 ml. mark, made strongly alkaline with 5 ml. ammonium hydroxide (0.88) run in slowly from a burette; 10 ml. cyanide-citrate-ammonia solution are now added and, after mixing, exactly 5 ml. chloroform is run in accurately from a burette followed by the addition of approximately 0.1 ml. of the 0.1 per cent. dithizone reagent. The test tube is stoppered with a well-washed rubber bung, shaken vigorously and the chloroform layer allowed to separate out. If this is bright red, indicating excess of lead, further additions of 0.1 ml. dithizone are made until excess of reagent is present, as shown by a green, blue or purple colour in the chloroform layer and a yellow tinge in the aqueous layer. The supernatant aqueous layer is removed by capillary tube (Pasteur pipette) using gentle suction from a water pump or syphon and the chloroform layer washed with two successive 20 ml. portions of cyanide-ammonia solution. The chloroform layer is passed through a Whatman No. 50 7 cm. filter paper into a small dry test-tube. The extinction coefficient is determined in a spectro-photometer, using in the compensating cell a "reagent blank" on the whole procedure. Reference to a calibration curve previously prepared with dilute standard lead solutions enables the concentration of lead in the sample to be read off direct. Since exactly 5 ml. of chloroform were used and the measurement depends solely on the intensity of colour, losses of coloured chloroform during washings and rapid filtration are immaterial.

The colour in chloroform is fairly stable in diffuse daylight, but fades rapidly in bright sunlight. Absorption is near the maximum at $528\text{ m}\mu$, corresponding to filter "S53" for the Pulfrich Photometer, but other filters can be used for higher quantities with relatively little loss of accuracy. Over the range which is normally covered, the relation between extinction coefficient and quantity is linear, so that multiplying the observed extinction coefficient by the reciprocal of the slope of the standard calibration curve gives the quantity present in the sample. Where large numbers of determinations are being made it obviously saves time to prepare a graph showing a variety of straight lines corresponding to variations in the quantity of material taken, dilutions used, stratum thickness, or light filter used, plotted against "p.p.m. in sample" on one co-ordinate. For very rapid approximate estimations numerous variations can be introduced, based on observations made at the stage of addition of reagent, without going back to the combustion phase.

Five g. of fresh tissue, 5 ml. of blood, and up to 2 g. of dried faeces or ground feeding stuffs can be dealt with in this manner. The relatively large amount of silica in cattle and sheep faeces does not cause interference, but for materials very high in calcium and phosphorus, or for fluids troublesome to concentrate by evaporation, modifications of the basic technique are used. Where the quantity of lead is very low and it is desired to use larger amounts of sample,

successive small moieties can be added to the same tube towards the end of combustion. For example, with normal sheep blood containing about 0.2 mg. per litre Pb, the combustion of 5 ml. is taken almost to completion, a further 5 ml. is added along with a further 2 ml. of perchloric acid, and the nitric oxidation repeated.

With the reagents commonly used in this laboratory the "blank" of the method is about 2 μ g. This is good enough for all ordinary analyses, but with normal blood a 5 ml. aliquot only contains 1 μ g. Pb or half the magnitude of the blank. The experimentally determined error is then about ± 20 per cent. With 10 ml. of blood this is reduced to ± 10 per cent. If a greater stratum thickness is adopted to secure greater sensitivity, say, by using the micro-cell of the Zeiss photometer which has a stratum thickness of 50 mm. for a volume of 1 ml., the scale reading of the blank becomes too high and nothing is gained unless the reagents are further purified. For special work, involving measurement for variations of very small magnitudes, this further purification can be effected by various procedures but is very troublesome.

MODIFICATIONS

Materials High in Calcium and Phosphorus

In materials such as bone the amount of calcium phosphate present is greater than that which can be held in alkaline solution by the amount of ammonium citrate used. The deposit of calcium sulphate at the end of the combustion procedure is also higher than that which can be taken up by reasonable amounts of citrate.

The second difficulty is avoided by omitting sulphuric acid from the combustion procedure and using perchloric and nitric acids alone, along the lines of the method adopted by Allcroft and Green (1935) for arsenic in biological materials. The first difficulty can be obviated by interposing a preliminary extraction in acid solution, along the lines of the diethyldithiocarbamate method of Tompsett and Anderson (1935).

Procedure.—The requisite quantity of material (up to 2 g. of dried fat-free ground bone) is combusted as usual by substituting 5 ml. of perchloric acid for 1 ml. of sulphuric and 3 ml. of perchloric acids in the "general method," and oxidising organic matter at the expense of nitric acid added *guttatim* as usual. The completed acid digest is diluted with distilled water while *still hot* and washed into a separating funnel to a volume of about 50 ml. To this is added 5 ml. of 2 per cent. aqueous solution of diethyldithiocarbamate, and 5 ml. of ethyl ether. After vigorous shaking and settling, the ethereal layer is run back into the combustion test-tube and a second 5 ml. portion of ether used to wash the solution. The ethereal washings are added to the first ethereal extract in the test-tube, and the ether evaporated by immersion in a water-bath. The dry residue is then digested in the usual way with sulphuric, per-

chloric and nitric acid and the determination completed as under "general method."

If it is desired to use a larger sample of whole or roughly broken bone, or the complete bone of a small laboratory animal, and evade the trouble of grinding to powder, the dried material may be de-fatted in a Soxhlet, weighed and dissolved in hot concentrated nitric acid under a reflux condenser. When solution is complete, this preliminary digest can be diluted to known volume and a suitable aliquot taken for completion of the combustion procedure given under "general method." Alternatively a sample of bone of any size can be dry-ashed in a muffle furnace at a temperature of $+50^{\circ}$ to 500° C., which does not involve loss of the lead phosphate in the bone, the ash dissolved or extracted with nitric or hydrochloric acid, diluted to known volume and an aliquot taken for the modified method above.

For milk the wet perchloric-nitric combustion tends to be a lengthy procedure because of the large amount of fat present. Schwaibold and Lesmuller (1939) found dry ashing in a muffle furnace to be more convenient and rapid. The ash can then be dissolved in acid and extracted with diethyldithiocarbamate as for bone, prior to completion of the dithizone procedure.

Urine

Since excretion of lead by the kidney is small and normal samples of urine contain exceedingly little, large samples have to be taken. These present mechanical difficulties both in wet combustion and in dry combustion, so that the calcium co-precipitation technique suggested by Ross and Lucas (1935) is preferable.

Procedure.—A 50 ml. sample of urine in a large centrifuge tube is acidified to litmus paper with 10 per cent. acetic acid; 2 ml. of 10 per cent. calcium chloride solution and 5 ml. saturated ammonium oxalate are then added. After mixing and allowing to stand for *at least two hours* to complete precipitation, the tube is centrifuged for 15 minutes at 3,000 r.p.m. and the supernatant fluid decanted or removed by suction. The precipitate is quantitatively transferred to the usual 8 in by 1 in. combustion test-tube, using successive small quantities of nitric acid—3 ml. to 5 ml. in all, including solution and washing; 2 ml. of perchloric acid are then added and combustion continued until about 1 ml. of residual perchloric acid remains. Subsequent procedure then follows the dithizone method for tissues and blood without the mediation of diethyldithiocarbamate. The lead is co-precipitated completely with the calcium oxalate and subsequent combustion is very easy.

Other Materials

The method obviously lends itself to a large variety of materials, with or without such special preparatory procedures, as may suggest themselves in special cases. For example, the lead in plumbiferous

soil dust which contaminates pasture grass in the vicinity of old lead-mining areas registers almost completely in the "general method," since galena and its weathered derivatives are broken down by mixed acid combustion. Contaminant lead is, of course, not distinguished from lead within the biological tissues themselves. The method is also suitable for general toxicological work, especially where the quantities of lead present are very small.

ACCURACY OF THE METHOD

The method makes it possible to determine directly the range 2μ to 40μ Pb with comfort by variation of stratum thickness and judicious use of filters in the final photometric comparison. Larger quantities are dealt with by dilution of a duplicate acid digest and selecting appropriate aliquots. If a duplicate digest is not available and the serial additions of 0.1 ml. dithizone show an unexpectedly large amount of lead, sight addition of further 5 ml. quantities of chloroform can be used to bring the colour within the range of the filters. This, of course, involves some loss of accuracy unless calibration against standards has also been made with varying amounts of chloroform.

A few recovery data are recorded in Table I.

TABLE I
RECOVERY OF LEAD ADDED TO STARCH AND TO FAECES,
URINE AND TISSUES OF SHEEP

Lead added μg .	Lead found					
	Starch (2 g.)		Blood (5 ml.)		Liver (5 g.)	
	Actual μg	Percentage	Actual μg	Percentage	Actual μg	Percentage
Nil	0.0	—	1.0	—	1.3	—
10	10.2	102.0	10.3	103.0	11.3	100.0
20	19.8	99.0	21.0	100.0	21.6	101.5
100	98.0	98.0	97.5	96.5	98.8	97.5

Lead added μg .	Lead found					
	Dried faeces (2 g.)		Urine (50 ml.)		Bone (4 g.)	
	Actual μg	Percentage	Actual μg	Percentage	Actual μg	Percentage
Nil	11.9	—	3.3	—	32.0	—
10	22.0	101.0	13.0	97.0	41.5	95.0
20	32.1	101.0	24.5	106.0	51.0	95.0
100	111.5	99.6	102.5	99.2	135.0	103.0

It will be noted that with one slight exception (urine plus $20\mu\text{g}$), recoveries all lie within ± 5 per cent., and this may be taken as the general accuracy of the method on individual samples of material.

Since the error of duplicate analyses of tissues, body fluids or excreta necessarily involves a small sampling error as well, the accuracy of the method *per se* is best expressed in terms of the variance of duplicate standards. Expressed as a coefficient of variation ($\delta_m \times 100$) the error of the method is ± 3.2 per cent. when the mean quantity estimated is 10 μg . Errors are naturally relatively greater when smaller quantities are estimated and when the "blank" is high in relation to the total photometric reading.

SUMMARY

A rapid general method for the determination of lead in biological materials is described, in which almost the entire operation is carried out in an 8 in. by 1 in. pyrex test-tube. Minor modifications are given for urine and for materials, such as bone, rich in calcium phosphate.

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LEAD AS A NUTRITIONAL HAZARD TO FARM LIVESTOCK

II. THE ABSORPTION AND EXCRETION OF LEAD BY SHEEP AND RABBITS

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INTRODUCTION

THE metabolism of lead in farm livestock is of interest for three reasons. First, a large number of farm animals die every year as the result of acute lead poisoning, this mortality being particularly large in the case of young calves (Robb and Campbell, 1940; Shrewsbury, King, Barrick, Hoefer and Doyle, 1944). Secondly, although chronic lead poisoning is a well-recognised entity in human beings, (Minot, 1938; Cantarow and Trumper, 1944), poor health or reduced efficiency of farm animals due to the continued ingestion of small quantities of lead has seldom been recorded (Gabel, 1941). Lastly, it has been suggested that lead may be a factor in the etiology of "swayback," a demyelination disease of lambs (Bennetts and Chapman, 1937; Innes and Shearer, 1940), and such a view might receive support from the high lead content of soils and pastures in some areas where the disease occurs (Shearer and McDougall, 1944). The available experimental evidence relating lead intoxication to the disturbance of copper metabolism of the ewe in "swayback" indicates that no such relationship exists (Eden, Hunter and Green, 1945), but the investigations of this disease have indicated a paucity of knowledge on the metabolic fate of lead in farm animals.

In order to obtain information on the importance of lead as a nutritional hazard to farm animals, investigations have been made in this laboratory on the absorption, excretion, retention and general metabolic and pathological effects of lead in both cattle and sheep, using the rabbit to explain certain aspects of the problems encountered. The papers which follow summarise the results of this investigation, while the final communication of the series attempts an appraisal of the importance of lead as a nutritional hazard and deals with the diagnosis of lead poisoning from the analyses of excreta, tissues and from the general disturbance of the animal's well-being.

The present paper deals with the absorption of lead from the digestive tract of sheep and its subsequent excretion in urine and faeces, together with confirmatory experiments using rabbits. The literature on these aspects of lead metabolism as they apply to the human and to laboratory animals has been amply reviewed by Sollman (1942) and by Cantarow and Trumper (1944).

THE ABSORPTION OF LEAD FROM THE DIGESTIVE TRACT

To what extent lead is absorbed from the digestive tract can be determined in three ways. First, an increase in blood levels of lead may be shown after lead ingestion; secondly, a comparable increase may be demonstrated in the concentration of lead in the tissues; and, lastly, absorption can be measured as the difference between the intake of lead in the food and its output in the faeces. All three methods have been used and it is therefore desirable to consider briefly their limitations.

An increase in systemic blood levels gives little quantitative information, for the concentration of lead found represents a complex balance between processes of absorption, deposition, mobilisation and excretion. Total carcass analysis (Tompsett, 1939; Shields, Mitchell and Kieth, 1939; Shields and Mitchell, 1941) excluding lead in the contents of the digestive tract, tend to estimate retention rather than absorption, for losses through re-excretion are ignored. Estimates by analysis of food and faeces ignore the active excretion of lead into the digestive tract or its more passive loss via the digestive secretions, but are closer to real estimates of absorption in so far that the urinary loss is not involved in the calculation. If carcass analyses are made relatively soon after dosage or exposure, urinary loss will be at a minimum and the two quantitative estimates should be in fair agreement, but both will tend to be underestimates owing to the excretion of lead by the digestive tract, it having been clearly demonstrated that the liver plays an active part in lead excretion (Annuschat, 1877; Aub, Fairhall, Minot and Reznikoff, 1926; Behrens and Baumann, 1933). In view of these facts, the values determined in the following experiments must be termed "apparent absorptions" rather than "true absorptions." The extent of the difference between apparent and true absorption is dealt with in the discussion.

Apparent Absorption Estimated from Food—Faeces Analyses

Ten experiments were carried out with sheep in which faeces were collected quantitatively while the sheep were being fed a constant ration. Each was conducted in the following manner. The sheep was confined in a metabolism cage and fed a constant quantity of food daily over a period of at least a week. Faeces were then collected, using harness of the design of Watson and Horton (1936) for a period of at least four days, generally much longer. Analyses of excreta and food were made daily during this period, using the method of analysis previously described (Blaxter and Allcroft, 1950). From analysis of variance of the results of a 12-day collection period it was found that the coefficient of variation attached to a single day's excretion of lead was 7.9 per cent. This is higher than the error attached to the method of lead estimation used and is largely accounted for by the day to day variation in faecal dry matter excretion by the sheep. The mean excretion over a five-day period

has a coefficient of variation of only 3.5 per cent., and even this error is probably an over-estimate owing to the fact that a low excretion of dry matter on one day is subsequently followed by a high excretion the following day, faecal excretion being under partial voluntary control. It follows, however, that the error attached to a small apparent absorption can be quite high, for even if lead were given as standard solutions, the error attached to the faecal excretion is considerable.

Table I summarises the results of the experiments. In view of the errors attached to the estimates as discussed above it is clear that within the range of 2 mg. to 110 mg. there is little change in

TABLE I
THE APPARENT ABSORPTION OF SMALL QUANTITIES OF LEAD BY SHEEP

Sheep No.	Daily ration in g.					Intake of Pb in mg. per day	Daily faecal output of dry matter in g.	Pb content of dry faeces mg. per kg.	Excretion of Pb	
									Mg. per day	Per- centage of intake
A	250	oats,	600	hay	...	1.9	257	7.6	2.0	102.6
B	200	"	776	"	...	2.4	297	7.5	2.2	94.5
C	200	"	780	"	...	2.4	325	8.7	2.8	118.8
C	200	"	800	"	...	2.1	293	5.8	1.9	90.4
B	800	hay ex	Derbyshire	...	...	11.5	328	34.3	11.3	98.3
C	800	"	"	...	...	11.5	312	36.9	11.5	99.3
B	200	oats,	790	hay,	50 mg. Pb. (acetate)	52.3	293	170.3	49.8	95.1
C	200	"	798	"	50 " (")	52.4	288	175.6	50.6	96.6
D	300	"	800	"	50 " (nitrate)	50.4	310	152.6	47.3	94.6
B	800	Derbyshire	hay,	100 mg. Pb	...	...	...	...	...	...
		(acetate)	...	...	...	115.5	352	306.8	108.2	97.0

the percentage of lead which is apparently absorbed. Similarly, it would appear that there are no outstanding differences between the apparent absorption of lead when given as acetate and as nitrate or when given in the form of naturally contaminated hay. Regardless of food source or amount of lead given within this range, the mean excretion was 98.7 per cent. This gives an estimate of absorption of 1.3 per cent. of the lead ingested, a value which has a standard error of ± 0.8 per cent.

Apparent Absorption Estimated from Blood Lead Concentrations

Five experiments were carried out in which blood samples were analysed following the absorption of lead from the digestive tract. It was necessary to use quite high quantities for this purpose in order to demonstrate peaks in absorption, for only very slight increases could be detected in sheep given 50 mg. or 100 mg. daily. In these experiments sheep fed normal rations were forcibly dosed

with lead compounds dissolved or suspended in water, blood samples being taken at intervals before and after dosage. From Table II and Fig. 1 it is clear that in the case of massive doses of lead the maximum blood level was reached at the 4th to the 6th hour following dosage. This finding confirms the observations on the slow rate of absorption of lead from the gastro-intestinal tract as reviewed by

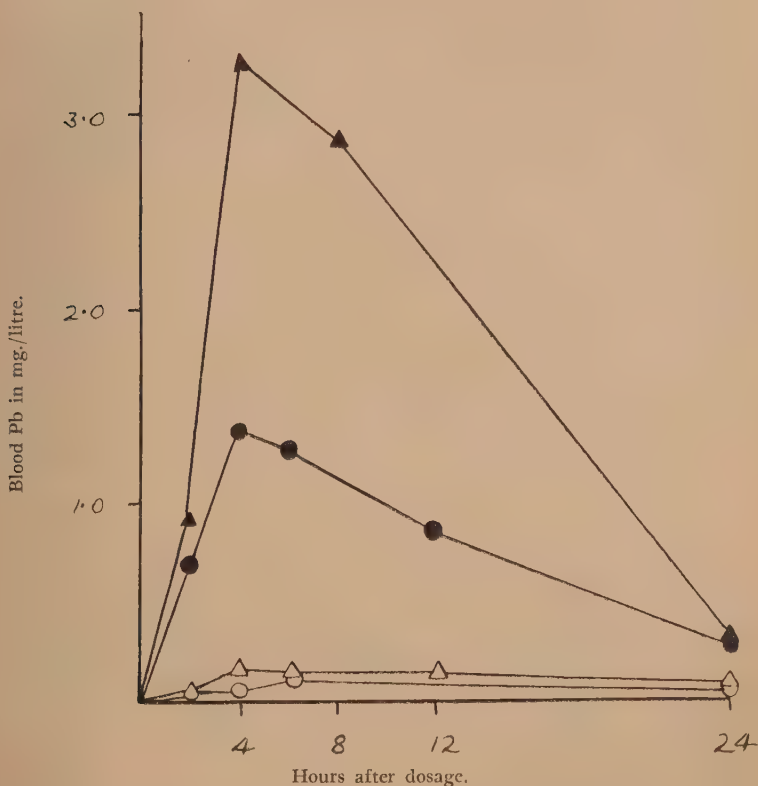


FIG. 1.—The effect of single doses of Lead Acetate on the Lead content of the whole blood of sheep.

Key

Solid triangles = 40g. Pb.	Open triangles = 20g. Pb.
Solid circles = 30g. Pb.	Open circles = 10g. Pb.

Cantarow and Trumper (1944) and is contrary to the results obtained when similar large doses of copper were given orally (Eden and Green, 1939) where a maximum occurred within the first hour of dosage. A finite estimate of absorption is impossible, but from the weight of the sheep their blood volumes may be calculated and the amount of lead circulating at the time of maximum blood lead

TABLE II
THE CONCENTRATION OF LEAD IN THE BLOOD OF SHEEP GIVEN LARGE
DOSES OF LEAD BY MOUTH

Sheep No.	Amount of lead given, g.	Blood lead in mg. per litre, hours after dosage						
		0	2	4	6	12	24	72
L/1	10, as acetate ...	0.08	0.09	0.12	0.21	—	0.12	—
F	20, " ...	0.11	0.08	0.26	0.21	0.25	0.31	0.19
G	30, " ...	0.08	0.78	1.46	1.37	0.94	0.63	0.40
L/A	40, " ...	0.12	1.05	3.40	2.96	—	0.59	—
C*	10, as basic carbonate	0.69	0.71	0.81	0.93	1.31	1.21	1.22

* This sheep had previously received 200 mg. of lead as the acetate intravenously accounting for the high preliminary figure.

concentration may be ascertained. In the case of sheep L/A given 40 g. of lead, 13.6 mg. were circulating at the time of maximum absorption, and in sheep G given 30 g. the figure was 5.8 mg. In view of the very slow rate at which lead leaves the blood stream (Blaxter, 1950; Allcroft, 1950) the absorption rate must indeed be small.

Apparent Absorption Estimated from Carcase Analyses

Two sheep died at comparable times after receiving large quantities of lead. One had been injected intravenously with 400 mg. of

TABLE III
ESTIMATED ABSORPTION OF LEAD BY THE SHEEP, AS ESTIMATED FROM CARCASE
ANALYSIS OF SHEEP POISONED BY VEIN AND BY MOUTH

Tissue	Pb content in mg. per kg.		Concentration of Pb in Sheep E, per 100 mg. of Pb injected	Mg. of Pb absorbed by Sheep L/A, assuming that distribution of Pb is the same as in Sheep E	Calculated percentage of Pb absorbed by Sheep L/A
	Sheep E given 400 mg. by vein	Sheep L/A given 40 g. by mouth			
	(1)	(2)	(3)	(4)	(5)
Brain ...	2.0	2.1	0.500	402	1.0
Muscle ...	0.3	0.3	0.075	426	1.1
Liver ...	75.6	37.9	13.900	201	0.5
Kidney ...	118.0	195.8	29.500	614	1.7
Lung ...	27.2	12.3	6.800	181	0.5
Adrenal ...	2.4	2.1	0.600	350	0.9
Skin ...	1.2	2.5	0.300	833	2.1
Colon wall ...	2.5	5.4	0.625	864	2.2
Mean ...					1.25

Column 4 is calculated by dividing the values in column 2 by the corresponding values in column 3.

Column 5 is calculated from column 4 by dividing each value by 400.

lead and the other had received 40 g. of lead as the acetate by mouth. These data on toxicity themselves provide an estimate of absorption, for it has been found that the lethal dose of lead by vein is between 200 and 400 mg., while the lethal dose of lead given as a single oral dose is between 30 and 40 g., suggesting that 1 to 2 per cent. of the lead ingested reaches the tissues in a sufficiently large quantity to cause death. Table III records the tissue concentration of lead in the two sheep and estimates of the percentage absorption of lead by the sheep which died as the result of oral poisoning. The method of calculation is clear from the table and it will be noted that the calculated percentage absorptions given in the last column show considerable variation. This is largely due to a differential distribution of lead as between liver and lung in the sheep poisoned by injection, and kidney and gut wall in the sheep poisoned by mouth. The reason for this is discussed in the following paper, but despite this variation it is obvious that absorption is in the region of 1 to 2 per cent. of the quantity ingested.

Similar but far more complete evidence of absorption using this method have been obtained in rabbit experiments. This is developed in Table IV where the lead concentrations in rabbit brain and skeletal muscle are related to the quantities of lead ingested or injected four days previously. The calculations of absorption given in the table were arrived at as follows. For the rabbits which were injected with lead the simple regression of concentration in the tissue on amount administered was calculated:—

$$x = bD + c.$$

where x = the tissue concentration of lead.

D = the amount of lead introduced into the circulation.

b, c = constants.

There was no indication of departure from linearity in the case of these equations. Lead absorption was then calculated by rearrangement of the terms of the above equation and applying it to the tissue concentrations found in the orally dosed rabbits:—

$$D = \frac{x - c}{b}$$

thus making the assumption that lead absorbed from the digestive tract is taken up by brain and heart muscle to the same extent as when lead is introduced into the general circulation.

The results show that in the rabbit, fed a normal stock diet, lead absorption is also relatively small, for the mean apparent absorption based on brain lead concentration was 1.0 per cent. and based on heart muscle lead concentration 0.8 per cent. These data would suggest that lead absorption in the rabbit is also very small when these large doses of lead are given by mouth.

It should be noted that this comparative method of serial slaughter does estimate true absorption rather than apparent absorption or retention. Processes of excretion are taking place in both

TABLE IV

ESTIMATED ABSORPTION OF LEAD BY RABBITS AS ESTIMATED BY CARCASE ANALYSES OF RABBITS POISONED BY VEIN AND BY MOUTH

Intra-venous dosage in mg.	Lead in mg. per kg.		Oral dosage in g.	Lead in mg. per kg.		Calculated absorption in mg. calculated on the basis of lead content of	
	Brain	Heart muscle		Brain	Heart muscle	Brain	Heart muscle
0	0.00	0.06					
5	0.55	0.80					
10	0.60	1.30	1.0	0.53	0.60	9.3	6.9
20	0.90	1.12	2.0	0.89	0.80	22.6	11.1
30	1.19	1.86					
40	1.34	2.27	4.0	1.22	1.55	34.8	41.6
60	1.80	3.54					

Regression equations: Brain: $C\ 0.28\ 0.027\ D$; Heart muscle: $C\ 0.25\ 0.051\ D$.

animals and it can be assumed, though it is not necessarily correct, that they are quantitatively very similar. If this is the case, then the relative tissue concentrations will in fact measure true absorption. In the present case the data suggest that the apparent absorption estimates which have been made are not grossly different from the true absorption of lead from the gut.

In view of these data indicating only a small apparent absorption of lead from the digestive tract it is of some interest to find in what form lead is excreted after its administration as a soluble salt. A sample of faeces from a sheep, which had been fed lead acetate, was extracted with water, dilute acids and ether with the results shown in Table V. This table indicates that very little lead is present as ether-soluble lead soaps, and little is present as carbonate or bound to protein, for this would have been extracted with dilute acetic acid. Most of the faecal lead can be extracted with dilute nitric acid, suggesting that the faecal lead is mostly present as phosphate or sulphide.

THE EXCRETION OF LEAD WHICH HAS BEEN ABSORBED BY THE BODY *Excretion in Urine and in milk*

While faecal lead represents unabsorbed lead with a variable admixture of re-excreted lead, urinary lead must represent lead

TABLE V
RECOVERY OF LEAD FROM FAECES

Extract	Lead found, mg. per kg.	Percentage of lead present found
Cold water	20	6.8
" 0.1 N acetic acid	67	23.0
" 0.1 nitric acid ...	280	96.2
Hot ether	1	0.3
Lead present in sample	291	100.0

TABLE VI
THE URINARY EXCRETION OF LEAD BY SHEEP

Sheep No.	Daily ration	Previous history	Lead ingested daily, mg.	Urine volume, ml.	Urine lead content, mg. per litre	Total daily urinary excretion, mg.
B	800 g. hay	Prior to exposure	2.1	983	0.07	0.07
E	800 " and 300 g. oats	" "	2.0	853	0.08	0.07
C	800 " " 250 "	" "	1.8	1,196	0.08	0.09
I	600 "	" "	2.0	372	0.09	0.04
B	200 g. oats and 800 g. hay	Following exposure 3 weeks previous	2.4	979	0.18	0.18
C	Normal ration plus 50 mg. Pb	First exposure	52.4	610	0.20	0.12
B	800 g. hay ex Derbyshire	" "	11.5	642	0.30	0.19
B	800 " " and 50 mg. Pb	Following ration of Derbyshire hay alone	111.5	970	1.27	1.23*
D	Normal ration plus 50 mg. Pb	After 4 months on normal ration	50.4	1,082	0.11	0.11
1F	" and 100 mg. of Pb	On same ration including Pb for 200 days	105	620	0.40	0.25
2F	" " 200 "	" " " 200 "	205	422	0.81	0.34
3F	" " 300 "	" " " 200 "	305	485	0.69	0.34
F	Normal ration	12 days following a single dose of 20 g. of Pb as acetate		557	0.50	0.28
G	"	14 days following a single dose of 30 g. of Pb as acetate		696	0.43	0.30
C	" but no food consumed	3 days following a single dose of 10 g. of Pb as basic carbonate		940	0.50	0.47
	Man, K.L.B.	No exposure			0.03	
	" G.S.	"			0.04	

* Contamination suspected.

which has been absorbed. To obtain information on the amounts of lead excreted in the urine, sheep were confined in wooden metabolism stalls and urine collected quantitatively, using a well-scrubbed rubber urine funnel attached to the washed belly of male sheep. Collections were made over periods of at least five days, care being taken to prevent contamination. No preservative was used in the urines. The urinary concentration of lead in normal sheep and in sheep given varying quantities of lead are shown in Table VI. The two values for human beings which are included are in agreement with normal values as quoted by Kench (1940) and reviewed by Cantarow and Trumper (1944). It will be noted that the values for four normal sheep are all higher than these two values, a fact that is understandable in so far that normal human diets seldom contain as much as 1 mg. of lead. Following lead ingestion the lead content of sheep urine is in every case higher than these normal low values, and in the case of sheep C when given 10 g. of lead as the basic carbonate, the lead content was seven times the normal value.

TABLE VII
THE LEAD CONTENT OF MILK SAMPLES FROM EWES AND A COW

<i>Animal No.</i>	<i>Treatment given</i>	<i>Day of lactation</i>	<i>Pb in mg. per kg.</i>
Cow 1 ...	None	Late	0.02
Ewe 1158	"	2	0.15
" 1150	"	2	0.11
" 1160	Dosed with 5 g. of lead as the acetate 7 days before lambing	2	0.28
		4	0.18
		6	0.15
" 1158	Given 100 mg. of lead as the acetate commencing 6th day of lactation	7	0.38
		14	0.55

Lead may also be excreted in the milk and this, too, will represent lead which has been absorbed. Only a small number of milk samples have been analysed. The results are shown in Table VII. All the samples were taken into clean glass beakers, care being taken to prevent contamination. The results show that in the ewe following lambing there is a relatively high excretion of lead much higher than has been reported in either the cow (Kehoe, Cholak and Story, 1940) or in women (Bagchi, Ganguly and Sirdar, 1940). The single value for a normal cow is in agreement with the data for the human. The high values obtained in the case of the sheep poisoned with 5 g. of lead prior to lambing are of interest. This dose corresponds to nearly 100 mg. of lead per kilogram of body weight and the corresponding lead concentration in the milk was only 0.28 mg./kg. White, Clifford and Calvery (1943) have found 2.8 mg./kg., i.e., ten times this amount, in cows which had received 1.65 g. of lead or approximately 4.8 mg. per kilogram of body weight. Such a high concentration of lead suggests that the amount

of lead ingested by these cows was in fact larger than was calculated from the lead contaminated rations they had been fed. Lead in the milk increased by nearly 400 per cent. when a ewe was given 100 mg. of lead acetate daily, and this lends support to Bagchi's contention that infants can be poisoned by way of their mother's milk (1941).

TABLE VIII

THE EXCRETION OF LEAD IN THE FAECES AND URINE OF SHEEP E, FOLLOWING THE INTRAVENOUS INJECTION OF 400 MG. OF Pb AS THE ACETATE

Day		Faeces				Urine				
		Wet weight g.	% Dry matter	Dry matter g.	Pb in mg. per kg.	Total Pb mg.	Volume ml.	Sp. g.	Pb in mg. per kg.	Total Pb mg.
6 before	...	1,177	30.4	357.9	8.8	3.18	1,239	1.040	0.09	0.11
5	"	907	36.9	334.3	5.7	1.90	452	1.040	0.08	0.04
4	"	961	43.8	420.5	5.2	2.21	1,020	1.040	0.05	0.05
3	"	774	47.0	363.8	3.8	1.39	824	1.037	0.05	0.04
2	"	1,042	44.1	459.8	4.1	1.87	625	1.033	0.05	0.03
1	"	794	44.4	352.8	4.3	1.51	955	1.033	0.08	0.08
1st day										
1 after 6 hours		399	41.4	165.0	4.1	0.68	322	1.023	0.48	0.16
2nd										
6 hours		170	45.6	77.5	4.6	0.36	0			
12-24	"	357	42.4	151.3	12.9	1.96	960	1.023	0.97	0.93
2nd day										
0-12 hours		335	32.8	110.0	14.3	1.50	995	1.020	0.92	0.16
12-24	"	311	31.0	96.4	23.9	2.30	825	1.031	0.82	0.68
3 after	...	290	31.4	90.9	32.8	2.98	643	1.024	0.71	0.45
4	"	15	45.9	6.9	50.5	0.35	370	1.040	0.50	0.19
5	"	137	25.8	35.3	104.8	3.70	450	1.041	0.47	0.21
6	"	170	16.9	28.8	101.4	2.92	468	1.039	0.35	0.16
7	"	25	26.4	6.6	33.7	0.22	223	1.033	0.37	0.08
8	"	158	22.8	89.6	32.4	1.16	325	1.034	0.24	0.08
9	"	0					520	1.032	0.24	0.12
10	"	246	14.8	36.3	27.8	1.01	890	1.017	0.11	0.10

The Relative Importance of the Kidney and the Digestive Tract in the Excretion of Absorbed Lead

If lead is introduced into the general circulation, then its excretion into the gut and in the urine can easily be measured and the relative importance of the two routes of excretion assessed. For this reason five experiments were carried out in which lead was injected into the jugular vein, and faeces and urine analysed both before and after injection. These experiments all conformed to the same general plan and only one is reported in detail, while the others are presented in summarised form. Each sheep was confined to a metabolism cage on a constant diet prior to the injection, and faeces and urine collected daily. Lead was then injected and faeces and urine again collected until excretion had once more fallen to a low level.

The complete data for sheep E injected with 400 mg. of lead are shown in Table VIII, and graphically in Fig. 2. It is clear from the table that, following the injection, faecal excretion of dry matter fell precipitously. This was *later* followed by a cessation of food intake. A comparison of pre- and post-injection excretion must, therefore, take into account the fact that the food mass was no longer passing through the gut at a normal rate. The most reliable estimate of the total amount of lead excreted is given in Table IX where the assumption is made that the normal faecal lead of the

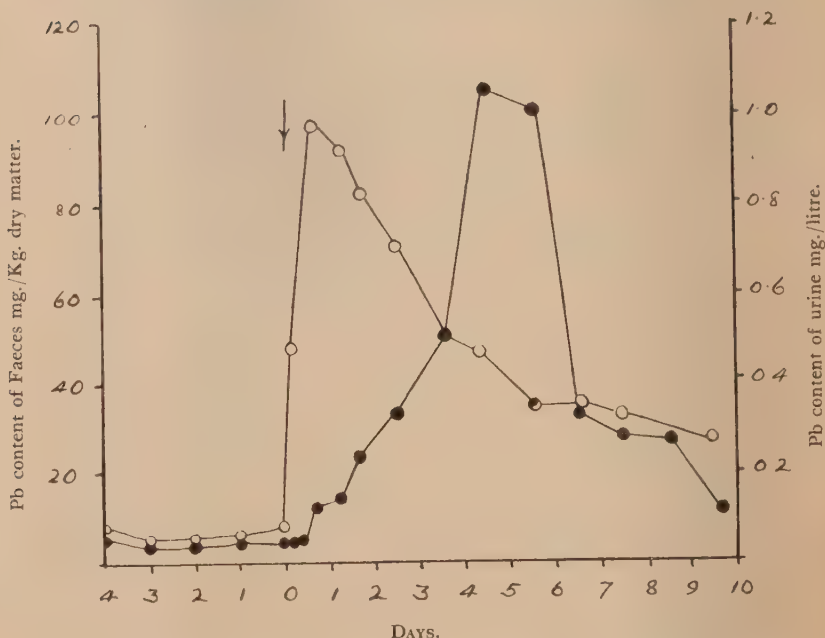


FIG. 2.—The effect of intravenous injection of 400 mg. Lead Acetate on the excretion of Lead in the urine and faeces of a sheep.

Key

Open circles=Urine Lead.

Closed circles=Faecal Lead.

experimental period is directly proportional to dry matter excretion. The results of the calculation show that of the 400 mg. injected, only 17.3 mg. were excreted over a period of ten days, and of this amount the larger proportion was in the faeces. Thus, of the total lead injected, only 0.7 per cent. was excreted by the kidney in a period of ten days.

The data obtained with other sheep using much smaller doses were essentially similar. The results summarised in Table X show

TABLE IX
THE INCREASE IN LEAD EXCRETION BY SHEEP E CALCULATED FROM THE DATA OF TABLE XII

Period	Total Pb in faeces in mg.	Total dry matter g.	Pb content of faeces mg. per kg.	Calculated normal faecal output of Pb in mg.	Increase in excretion of Pb in faeces in mg.	Total urinary output of Pb in mg.	Increase in urinary output in mg.	Total increase in Pb excretion in mg.
Control (6 days)	12.06	2,289.1	5.27	—	—	0.33	—	—
Days 1+2	4.50	503.8	—	2.66	1.84	1.93	1.81	3.65
3+4	5.28	97.8	—	0.52	4.76	0.64	0.53	5.29
5+6	6.62	64.1	—	0.34	6.26	0.37	0.26	6.52
7+8	1.38	96.2	—	0.51	0.87	0.16	0.05	0.92
9+10	1.01	36.3	—	0.19	0.82	0.22	0.11	0.93
Total 170	18.79	—	—	—	14.55	3.32	2.76	—

TABLE X
THE EXCRETION OF LEAD IN FAECES AND URINE FOLLOWING INTRAVENOUS INJECTION

Sheep No.	Amount of Pb injected mg.	Additional Pb found in urine in mg.			Additional Pb found in faeces in mg.			Total Pb recovery in 6 days		Percentage excreted Pb found in		Maximum concen- tration of Pb as a percentage of control period concentration	
		Days 1+2	Days 3+4	Days 5+6	Total	Days 1+2	Days 3+4	Days 5+6	Total	Faeces	Urine	Faeces	Urine
A	10	0.06	0.00	0.14	0.20	0.08	0.20	0.32	0.44	68.8	31.2	108	133
C	50	0.33	0.29	0.09	0.71	0.50	0.82	0.74	2.83	74.4	25.6	184	216
A	100	1.10	0.74	0.08	1.92	1.96	2.60	1.75	6.31	76.7	23.3	333	329
C	200	1.82	1.66	0.48	3.96	2.39	5.60	8.67	16.66	80.8	19.2	870	621
E	400	1.81	0.53	0.26	2.60	1.84	4.76	6.26	12.86	83.2	16.8	1,941	1,212

that only 4 to 10 per cent. of the injected lead could be recovered in the six days following injection. The highest value of 10 per cent. appeared to be largely due to the fact that this sheep doubled its urinary volume during the post-injection period. Of the lead recovered, the proportion in the faeces was not constant, but increased with increasing intravenous dosage. This is also illustrated by the last two columns of the table which indicate that the lead concentration in the faeces increased at a more rapid rate with increasing dosage than did the concentration of lead in the urine. These two facts suggest that small quantities of lead introduced into the circulation are largely eliminated by the kidney, while with larger quantities the gut is the more important vehicle of excretion.

From Fig. 2 and the roughly classified data of Table X it is clear that the peak concentration of faecal lead excretion occurs on the third to the fifth day following injection, while the peak concentration of urinary lead occurs on the first day. This suggests that the greater part of the intestinal excretion is in the upper part

TABLE XI
LEAD CONTENT OF COLON WALL, BILE AND LIVER IN SHEEP AND RABBITS

<i>Animal</i>	<i>History</i>	<i>Lead in mg. per kg.</i>		
		<i>Liver</i>	<i>Bile</i>	<i>Colon</i>
Sheep E ...	Died after 400 mg. Pb intravenously	75.6	2.4	2.5
L.A. ...	" 40 g. Pb orally	37.9	6.9	5.4
Rabbit 1 ...	No treatment	0.4	0.06	0.00
" 5 ...	Killed after 30 mg. Pb intravenously	145.2	65.5	2.56
" 6 ...	" 40 " "	211.7	53.4	3.76
" 7 ...	" 60 " "	316.0	28.8	4.10
" 8 ...	" 1 g. Pb. orally	19.2	13.5	2.36
" 9 ...	" 2 " "	54.6	3.5	11.76

of the gut, and the fact that the excretion in the faeces is delayed, only reflects the time taken for the food mass to traverse the distance from duodenum to rectum. It follows, therefore, that little lead is excreted in the lower part of the digestive tract. It might be expected that lead would also be excreted by the colon wall, for it has been claimed that calcium is excreted in this manner (Cowell, 1937), and in this respect it is of interest to compare the relative concentrations of lead in bile and colon wall in post-mortem material. This is presented in Table XI and shows that on a fresh weight basis the bile contains very considerable quantities of lead, while the colon wall is variable in its lead content and never very high, the last value in the table reflecting the difficulty of cleaning the digestive tract of its contents. It is obvious that secretion of lead through the wall of the colon is not a major factor in lead excretion.

Direct Confirmation of the Quantitative Importance of the Liver in the Excretion of Lead

Even though the literature shows that the liver is of great importance in excreting lead which has gained access to the circulation, and though the injection experiments dealt with above show that most of the lead excreted in the faeces of the sheep is derived from the upper part of the intestinal tract, direct evidence of biliary excretion is of considerable importance, for high intestinal excretion necessarily includes secretion in the succus entericus. A sheep was therefore operated on under nembutal anaesthesia and a small glass canula inserted into the common bile duct just below the gall bladder (Blaxter and Cowie, 1946). The bile was diverted to the outside of the body cavity by a tube sutured to the omentum thus providing continuous drainage comparable to that used by Schorregge (1933). Recovery from the operation was uneventful and realimentation of the sheep progressed well. For the purpose of the experiment the sheep was harnessed to collect urine and faeces, and the bile was drained continually into a clean receptacle through a rubber tube welded to the side of the urine funnel. Bile salts were given by mouth and after a preliminary period of four days, in which the normal routes of excretion were ascertained, 150 mg. of lead as the acetate were injected slowly into the jugular vein. Urine, bile and faeces were again collected quantitatively until the animal's death on the seventh day following the injection.

The results are summarised in Table XII. It will be noted that whereas the bile flow in the preliminary period was quite normal as judged by the results of Schorregge (1933) and Sobodtka (1937), following the injection bile flow increased by 100 per cent. This was associated with a fall in the dry matter content of the bile from 3.1 to 3.5 per cent. to 1.98 per cent., and an increase in total solids excretion in the bile from 15.4 to 15.9 g. per day to 23.8 g. This large increase in output was then followed by a fall to a value below the preliminary levels. Prior to the injection, urine volume was low and specific gravity high (1.051), and in the period after the injection anuria occurred, lasting from 60 to 100 hours. This was followed by diuresis, albuminuria and severe haemoglobinuria. Expected changes in faecal output occurred, but not the diarrhoea, which had been typical of the five previous injection experiments. It will be noted, however, that there was no peak in faecal lead excretion on the third to fourth day following injection, while a considerable early peak occurred in biliary excretion. This demonstrates that there is little excretion in the upper part of the digestive tract itself, the biliary excretion being the main cause of the faecal excretion in the five previous experiments. Calculations from Table XII indicate that of the lead injected, 1.27 per cent. was excreted in the urine, 5.31 per cent. in the bile and only 0.17 per cent. in the bile-free faeces. Of the total lead which was excreted (11.15 mg.)

TABLE XII

LEAD CONTENT OF WHOLE BLOOD, SERUM, BILE, URINE AND FAECES FOLLOWING THE INJECTION OF LEAD

Hours from injection	Urine		Total Pb, mg.	Volume ml.	Bile		Faeces		Total Pb mg.	Blood Pb mg. per l.	Serum Pb mg. per l.
	Pb mg. per l.	Volume ml.			Pb mg. per l.	Total Pb mg.	Total matter, g.	Pb mg. kg.			
Before											
96-72	290	0.09	0.027	490	0.30	0.148	280	8.54	2.39		
72-48	370	0.09	0.035	500	0.28	0.150	337	7.72	2.60		
48-24	420	0.08	0.036	485	0.28	0.136	304	5.57	1.69	0.19	0.04
24-0	410	0.12	0.052	455	0.32	0.146	233	4.97	1.16		
After											
0-3	50	5.15	0.257	161	3.61	0.581	165	4.90	0.81	{	34.5
3-6				157	4.40	0.691					6.2
6-12	120	4.02	0.482	251	5.35	1.343	67	4.27	0.29		
12-24					4.60	1.403	22.5	3.91	0.09		
0-24	170		0.737	874		4.018	255		1.19		
24-36	359	1.74	0.624	526	3.56	1.872	11.3	6.06	0.07	2.1	0.74
36-48	89	1.66	0.147	448	2.01	0.900	22.9	8.76	0.20		
24-48	448		0.871	974		2.772	34.2		0.27		
48-72	134	1.85	0.247	677	1.21	0.819	0.0				
72-96	0			545	1.16	0.632	1.8	15.1	0.03	1.1	0.11
96-120	355	0.28	0.099	370	1.22	0.451	7.4	15.7	0.11		
120-142	710	0.32	0.227	305	3.72	1.135	16.7	19.8	0.33	0.45	0.05
142-148	280	0.13		41	1.30	—	2.5	18.1			

17.1 per cent. was in the urine, 80.7 per cent. was in the bile and 2.2 per cent. was in the bile-free faeces.

It will be noted from Table XII that even three days after the injection of lead, the concentration of lead in the bile was always greater than the concentration of lead in the serum of the sheep. The gall bladder was intact in the sheep, but drainage was continual, and so it does not seem likely that concentration in the gall bladder can account for this difference. Similarly, the concentration

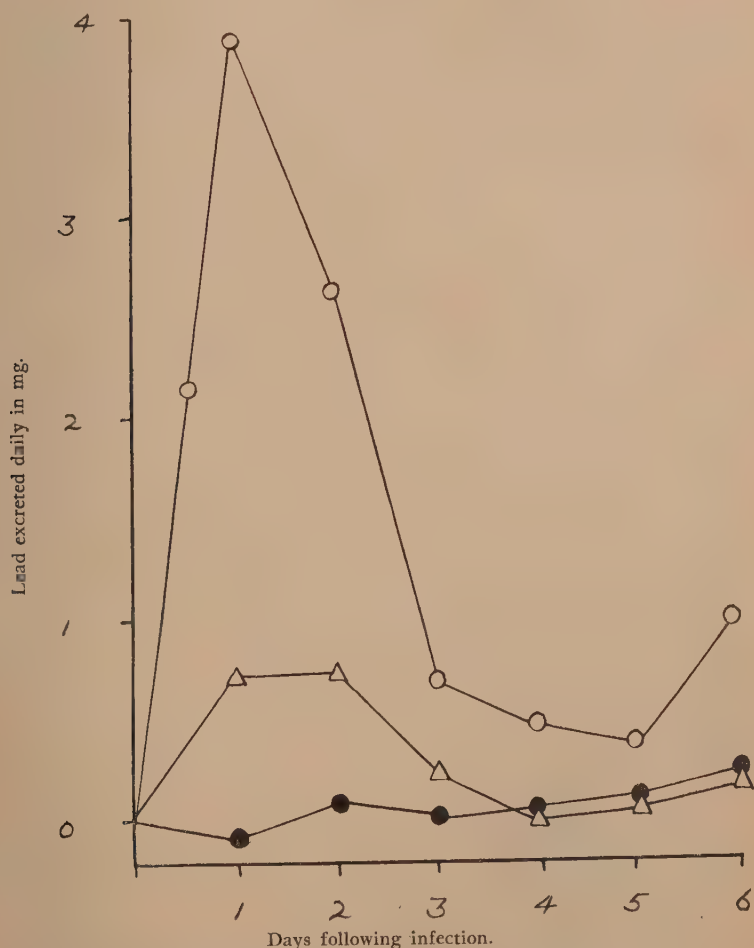


FIG. 3.—The total excretion of lead in urine, bile and bile-free faeces following the intravenous injection of 150 mg. Lead acetate.

Key

Open triangles = Urine.

Open circles = Bile.

Closed circles = Bile-free faeces.

of Ca in the bile was only slightly greater than the concentration of Ca in the serum, also suggesting no large concentration of the bile in the gall bladder. It seems probable, therefore, that lead is actively excreted by the liver and in view of the large quantities of protein which were present in the bile following the injection, it appears that the excretion of these large quantities involves the breakdown of the liver cells.

DISCUSSION

The estimates of apparent absorption of lead which have been made are all considerably complicated by the excretion of lead into the intestine. It has been suggested by Bricker (1926) that faecal lead consists of lead excreted by the intestine and only small quantities derived from the liver and pancreas. From the present results it would appear that the liver accounts for the major part of the lead excreted into the intestine of the sheep, there being but negligible quantities secreted by the walls of the colon or small intestine. This is in agreement with the studies of Behrens and Baumann (1933), who found that the high quantity of radio-active lead in the duodenum was all derived from the bile.

In order to estimate the true absorption of lead, the excretion by the liver must be discounted. From the evidence presented it is clear that re-excreted lead is not the major source of faecal lead, the major source being unabsorbed food lead. Thus in sheep I when consuming a ration containing only 2 mg. of lead daily prior to injection, 0.14 mg. of lead were excreted in the bile and yet nearly 15 times this quantity was excreted in the bile-free faeces. Even at this low level of lead ingestion, the re-excretion of lead is not a major factor. Similarly, blood levels of lead observed in sheep which had received massive doses are not so high as to suggest a large re-excretion by the liver. Further information relative to the quantitative importance of re-excretion is given by the injection experiments. Of the lead injected only 4 to 10 per cent. was excreted over a period of six days and of this only two-thirds to three-quarters could be traced to the bile. In the case of the lowest dose given—10 mg.—which was probably equivalent to 1 to 2 g. given by mouth, only 0.44 mg. was excreted in the faeces over a period of six days. This small quantity would not upset the apparent absorption data unduly.

It can be concluded therefore that over a considerable range of dosage—from 2 mg. to 40 g.—the apparent absorption data are not unduly complicated by the re-excretion of lead in the bile. True absorptions are, it is true, slightly higher than apparent absorptions, but the difference expressed as a percentage of the amount ingested is small. In terms of actual quantities, however, it is clear that as much as 3 to 4 mg. of lead can be excreted by the liver daily when large quantities of lead have been given.

In so far that the re-excretion of lead does not result in major

complications of the results presented in the first part of this paper, it is legitimate to compare the estimates of absorption which have been made. The ten sheep experiments indicate an apparent absorption of small quantities of lead (2 mg. to 100 mg. daily) of 1.3 ± 0.8 per cent. The tissue analyses of heavily poisoned sheep suggest an absorption of 1.2 per cent. The rabbit experiments in which amounts of lead equivalent to 15 to 50 g. in sheep were given, show an absorption of 0.8 to 1.0 per cent. These results are in substantial agreement and suggest only a relatively slight reduction of true absorption with increasing quantity ingested. They differ, however, markedly from those of Fees (1932), who found that over 90 per cent. of small amounts of ingested lead was absorbed by the intestine of the mouse within one hour. The absorptions obtained in the sheep are also much lower than the lead *retentions* found by Shields and Mitchell (1941) in rats fed rations containing varying amounts of Ca and P. The reason for these differences in absorption is not known, but their presence can for the moment be called a species difference in absorption.

The normal urinary level of lead excretion in the sheep appears quite comparable with the urinary excretion of lead by the human, if anything being slightly greater. This presumably reflects the greater intake of lead by the sheep even when fed so-called "normal" rations. It is evident that when lead ingestion is increased there is an increase in the lead excreted in the urine. There is not, however, a direct proportionality and in no case has more than 0.8 mg. been found in the urine of sheep, even when acutely poisoned. In the case of human beings, values as high as this have been reported where there has been severe occupational exposure to lead (Cantarow and Trumper, 1944).

It therefore follows that the retention of lead will increase as dosage increases. From the data in Table I and in Table VI the excretion of lead in faeces and urine may be predicted from the amount ingested, according to the two regression equations:—

Daily faecal lead excretion (mg.) = $0.02 + 0.964 \times (\text{mg. of lead ingested daily})$.

Daily urinary lead excretion (mg.) = $0.089 + 0.0012 \times (\text{mg. of lead ingested daily})$.

On summation of these two equations one can relate lead excretion to lead intake.

Total daily excretion of lead (mg.) = $0.109 + 0.9649 \times (\text{mg. of lead ingested daily})$.

The sheep will be neither retaining or losing lead when the intake is therefore:—

$$\text{mg. of lead to give a zero lead balance} = \frac{0.109}{1 - 0.9649} = 3.1 \text{ mg.}$$

These data suggest that no lead is retained if the sheep ingests less than 3 mg. daily. In this respect, various authors (see Sollmann,

1942; Cantarow and Trumper, 1944) have placed the lower limit of safety for lead ingestion in the human as from 0.2 to 2.0 mg. daily, and experiments over long periods of time with human beings have indicated that 1 mg. or 2 mg. of lead ingested daily does not give rise to large retentions or to the incidence of clinical symptoms of intoxication (Kehoe, *et al.*, 1940).

In the case of sheep existing on natural diets high in lead content, such as grazing sheep in Derbyshire and certain other lead-mining areas, the daily intake of lead over the whole year probably averages 20 mg. daily. From the above equation this intake would result in a daily retention of 0.59 mg., or over a period of three years, 570 mg. If all this lead was deposited in the skeleton, the concentration in the bones would be approximately 70 mg. per kg., a value within the normal range of concentration of lead in the bones of mature sheep from this area (Allcroft and Blaxter, 1950). This calculation provides a substantial confirmation of the accuracy of the equation. It can be concluded, therefore, that small lead retentions do in fact occur in sheep in these areas. Their interpretation in relation to chronic lead poisoning is dealt with in the final paper of this series (Allcroft and Blaxter, 1950).

SUMMARY

Experiments with sheep are described in which the faecal and urinary excretion of lead has been determined.

It is shown that the excretion of lead in the faeces consisted largely of unabsorbed food lead, and that within the range of intake of 2 mg. to 40 g. of lead, absorption was very small. The faecal lead was largely excreted as phosphate or sulphide.

The urinary excretion of lead is dependent on lead intake, but even when large quantities were given did not exceed 0.8 mg. daily. On low intakes (2 mg.) the urinary lead excretion was of the same order of magnitude as the excretion reported in the normal human.

Lead was excreted in the milk of the ewe in relatively large quantities when the animal had been given lead in its diet.

The bile is the major channel of lead excretion when large quantities are given, and the colon and wall of the intestine are of very little importance in excreting lead which has gained access to the body.

When less than 3 mg. of lead are ingested daily by the sheep there is no retention of lead. Above this quantity lead is retained in increasing quantities by the tissues. These results differ markedly from those on mice, rats, and humans.

The avidity of the tissues in retaining lead was shown by the fact that when lead was injected into the systemic circulation, only 4 to 10 per cent. of the amount injected was recovered over a period of six days following the injection.

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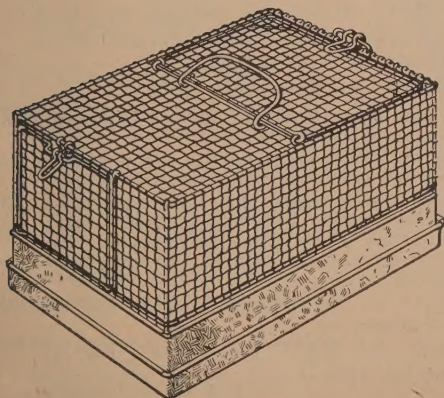
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